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Interaction of β-Lactoglobulin with κ-Casein in Micelles As Assessed by Chymosin Hydrolysis. Effects of Added Reagents

I. Mohan Reddy and John E. Kinsella*

Institute of Food Science, Stocking Hall, Cornell University, Ithaca, New York 14853

The effect of lysozyme, ovalbumin, retinol, sucrose, 1-anilino-8-naphthalenesulfonate (ANS), sodium sulfite, sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and cetylpyridinium chloride (CPC) on heat-induced interaction of β-lactoglobulin (β-Lg) with κ-casein (κ-C) on intact micelles was studied by following the kinetics of chymosin hydrolysis. Heating of β-Lg with casein micelles in Jenness–Koops buffer (pH 6.8) inhibited chymosin hydrolysis, resulting in decreased initial velocity (V_i) and glycomacropeptide (GMP) release. Lysozyme (0.4%), CTAB (2.6×10^{-3} M), and CPC (2.6×10^{-3} M) prevented the inhibition of chymosin hydrolysis, possibly by neutralizing the overall negative charge on casein micelles. On the other hand, SDS (5.2×10^{-3} M), ovalbumin (0.5%), and ANS (1.04×10^{-3} M) caused a further inhibition of chymosin hydrolysis. SDS and ovalbumin might have increased the net negative charge of the casein micelles, resulting in an increased electrostatic repulsion between the enzyme and substrate. Sodium sulfite (5.0×10^{-3} M) and sucrose (6.67%) prevented the inhibition of chymosin hydrolysis to some extent, whereas retinol (7.2×10^{-4} M) did not have any influence on hydrolysis. A concentration of 0.4% lysozyme prevented the inhibition of chymosin hydrolysis in heated skim milk and mixtures of casein micelles and β-Lg and restored the rennet clotting time of heated milk.

It is clearly established that β-lactoglobulin (β-Lg) and κ-casein (κ-C) form a heat-induced complex via thiol/disulfide (-SH/-SS-) exchange reactions and hydrophobic interactions in model systems as well as in milk (Sawyer, 1969; Elfagm and Wheelock, 1977; Snoeren and van der Spek, 1977; Dziuba, 1979; Smits and van Brouwershaven, 1980; Doi et al., 1981, 1983; Haque et al., 1987; Haque and Kinsella, 1988; Parnell-Clunies et al., 1988). It has been suggested that this complex formation is responsible for the increased clotting time and the reduction in total amount of peptides released by chymosin when model systems of casein micelles and β-Lg or milk is heated (Sawyer, 1969; Hindle and Wheelock, 1970; Wilson and Wheelock, 1972; Wheelock and Kirk, 1974; Shalabi and Wheelock, 1977; Mohan Reddy and Kinsella, 1990).

We recently reported a detailed study of the interaction of β-Lg with κ-C on intact micelles by following a complete time course of chymosin hydrolysis (Mohan Reddy and Kinsella, 1990). The effects of temperature, heating time, β-Lg concentration, and pH on the interaction was reported. The results indicated that the interaction, as observed by a decrease in initial velocities of chymosin reaction and the total amount of glycomacropeptide (GMP) released, reached a maximum after heating for 10 min at 85 °C. Concentrations of β-Lg higher than that present in milk (>0.32%) facilitated the interaction. Matterella and Richardson (1982) reported that positively charged β-Lg derivatives bind casein micelles strongly and decrease the electrokinetic potential as well as rennet clotting time of casein micelles. β-Lg carries a net charge of −10 at pH 6.6 (Basch and Timasheff, 1967). Apparently, the interaction of β-Lg with κ-C on intact micelles increases overall negative charge of the micelles and causes electrostatic repulsion between the chymosin and substrate. This may partly explain the inhibition of chymosin hydrolysis of κ-C on micelles following interaction with β-Lg. Therefore, it seemed appropriate to further study the effect of certain cationic and anionic detergents and positively and negatively charged proteins on the β-Lg–κ-C interaction by following chymosin hydrolysis. In addition, the effects of sucrose, which stabilizes β-Lg against heat denaturation (Garrett et al., 1988) and retinol and 1-anilino-8-naphthalenesulfonate (ANS), which bind to β-Lg and caseins, respectively (Hemeley et al., 1979; Fugate and Song, 1980; Sugimoto et al., 1974), on the interaction of β-Lg with κ-C warranted examination.

The present study investigated the effects of cetyltrimethylammonium bromide (CTAB), cetylpyridinium chloride (CPC), sodium dodecyl sulfate (SDS), lysozyme, ovalbumin, sucrose, sodium sulfite, ANS, and retinol on the interaction of β-Lg with κ-C on intact micelles by following kinetics of chymosin hydrolysis.

MATERIALS AND METHODS

Materials. Fresh skim milk (Holstein) was obtained from the Cornell Dairy Plant. Chymosin (activity approximately 60

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units/mg of protein), fluorescamine, retinol, sodium dodecyl sulfate, 8-anilino-1-naphthalenesulfonic acid, ovalbumin, cetyltrimethylammonium bromide, cetylpyridinium chloride, and lysozyme were purchased from Sigma Chemical Co., St. Louis, MO. Spectral-grade ACS-certified acetone was obtained from Fisher Scientific (Rochester, NY). ANS was recrystallized three times from hot water and used. All other chemicals used in this study were of reagent grade.

Methods. *β-Lactoglobulin* (a mixture of A and B variants) was isolated from fresh skim milk by the procedure of Armstrong et al. (1967), purified as described (Mohan Reddy and Kinsella, 1990), lyophilized, and stored at 4 °C under desiccation.

κ-Casein (κ-C) was isolated from freshly prepared acid-casein by the modified (Doi et al., 1979) procedure of Zittle and Custer (1963), purified as described (Mohan Reddy and Kinsella, 1989), lyophilized, and stored at 4 °C under desiccation.

Glycomacropeptide (GMP) was prepared from purified κ-C by treating with chymosin as described by Ono et al. (1987). After addition of chymosin to κ-C solution in 0.07 M NaCl, the mixture was incubated at 35 °C from 30 min and then centrifuged at 15000*g* for 30 min. The supernatant was fractionated by eluting with deionized water from a Sephadex G-75 column. The GMP fraction was collected and stored at −20 °C.

Casein micelles were prepared from raw skim milk (100 mL) by centrifugation at 78000*g* for 1 h at 20 °C in Sorval OTD-65B ultracentrifuge. The casein pellet was redispersed in simulated milk buffer (pH 6.8) (Jenness and Koops, 1962), which was slightly modified by increasing the sodium citrate content from 6.0 to 7.2 mM (Mohan Reddy and Kinsella, 1989). The casein micelle solution was centrifuged again, dispersed to homogeneity, and made to 75 mL in the same buffer at pH 6.8. The usual casein concentration was 30 ± 1 g/L.

Protein Solutions. β-Lg was dissolved in modified milk salt buffer (pH 6.8) and the mixture centrifuged at 20000*g* for 30 min. The concentration of β-Lg and κ-C in solution was determined spectrophotometrically at 280 nm of by using $E^{1\%}_{1cm}$ 9.5 and 10.0, respectively (Townend et al., 1964; Makino and Niki, 1977). GMP was determined by a microbiuret method at 310 nm (Itzhaki and Gill, 1964). The protein concentration of casein micelles was determined by micro-Kjeldahl nitrogen estimation. A factor of 6.34 was used to convert nitrogen to protein value (Karman and Van Boekel, 1986).

Additive Solutions. Ovalbumin, lysozyme, ANS, and sucrose were dissolved in modified milk salt buffer (pH 6.8). Sodium sulfite, CTAB, CPC, and SDS were dissolved in distilled water, and retinol was dissolved in ethyl alcohol.

Preparation of Reaction Mixtures. To 7.5 mL of casein micelle solution (approximately 30 mg/L) was added 1.0 mL of 3% β-Lg solution and the required quantity of additive solution, and the volume was made to 10.0 mL with modified milk salt buffer (pH 6.8). The final concentrations of total casein (approximately 2.25%) and β-Lg (0.3%) in the reaction mixture were approximately the same as in milk. The concentration of additives in the reaction mixture was as follows: retinol, 51.57 μg/mL (7.2×10^{-4} M); ANS, 0.31 mg/mL (1.04×10^{-3} M); sucrose, 6.67% (1.95×10^{-1} M); SDS, 1.50 mg/mL (5.20×10^{-3} M); CTAB, 0.94 mg/mL (2.6×10^{-3} M); CPC, 0.93 mg/mL (2.6×10^{-3} M); sodium sulfite, 0.63 mg/mL (5.0×10^{-3} M); ovalbumin, 0.5% (1.1×10^{-4} M); lysozyme, 0.1–0.4% (6.85×10^{-5} to 2.74×10^{-4} M). Addition of CTAB and CPC resulted in some precipitation of casein micelles. The concentration of alcohol in the reaction mixture containing retinol was kept below 1.0%. When skim milk was used for experiments, the additive dissolved in water was added to 9.0 mL of milk and the final volume made to 10.0 mL with distilled water.

Heat Treatments. Aliquots (10 mL) of the reaction mixture were heated in screw-cap tubes (25 mL) at 85 °C in a thermostatically controlled water bath. Each sample was preheated for 4 min to attain 85 °C and held at that temperature for 10 min. After heating, the tubes were cooled to 15 °C in water and stored overnight at 5 °C. Unheated reaction mixture or skim milk without any additive (unheated control) was also stored at 5 °C along with heated samples.

Chymosin Hydrolysis. To 10 mL of the reaction mixture, preequilibrated at 30 °C for 30 min in a water bath, was added 25 μL of 0.02% chymosin solution, and the rate of the enzyme reaction was determined on the basis of the release of GMP soluble in 8% trichloroacetic acid (TCA) (van Hooydonk and Oleiman, 1982). Aliquots (0.5 mL) of the reaction mixture after the addition of enzyme were withdrawm at regular intervals, and the reaction was stopped by vigorously vortex-mixing into 1.0 mL of a 12% TCA solution, resulting in a final TCA concentration of 8%. An aliquot of sample treated in the same way with TCA was taken before the addition of enzyme and used as a blank. TCA (8%) immediately inactivates chymosin and precipitates the casein and the whey proteins (van Hooydonk and Oleiman, 1982). After 1-h storage at 30 °C, the precipitated samples were filtered (Whatman 42) and the filtrates analyzed for GMP.

Estimation of GMP. GMP was routinely estimated fluorometrically at pH 6.0 by the fluorescamine method of Beeby (1980). An aliquot (100 μL) of the blank and sample was added to 3.0 mL of a sodium tetraborate solution (0.2 M) adjusted to pH 6.0 with concentrated HCl. One milliliter of a solution of fluorescamine in acetone (0.2 mg/mL) was added quickly with rapid vortex-mixing. After 15 min, the fluorescence was measured at 480 nm (excitation 390 nm) in ratiometric mode with a Perkin-Elmer 650-40 fluorescence spectrophotometer, using 1-cm-square cuvettes thermostated at 25 °C. The excitation and emission band widths were set at 5 nm.

HPLC analysis of 8% TCA filtrates (Mohan Reddy and Kinsella, 1989) revealed the presence of nonprotein nitrogen (NPN) along with GMP. Hence, the sample fluorescence readings were corrected for the NPN fluorescence by subtracting the respective blank values. A standard graph of GMP (μg) vs fluorescence at 480 nm was constructed with purified GMP. The concentration of GMP (μg) corresponding to corrected sample fluorescence was read from standard graph and expressed in micromolar units (μM). A molecular weight of 8000 is used for GMP.

RESULTS AND DISCUSSION

Effect of Sucrose, Retinol, and ANS. These were checked by heating samples of a mixture containing casein micelles and β-Lg in presence of sucrose (6.67%), retinol (7.2×10^{-4} M), or ANS (1.04×10^{-3} M) and measuring chymosin hydrolysis. The results of kinetics of hydrolysis (Figure 1; Table I) indicate that sucrose increased the V_i and the final amount of GMP released, whereas retinol did not influence the enzymic reaction. ANS decreased the chymosin hydrolysis, as indicated by V_i and GMP released. The data indicate that sucrose probably prevented the interaction of β-Lg with κ-C to some extent, whereas retinol did not influence the interaction at all.

The complex formation between β-Lg and κ-C involves hydrophobic interactions as well as intermolecular –SS– bonds (Sawyer, 1969; Dziuba, 1979; Smits and van Brouwershaven, 1980; Doi et al., 1983; Haque and Kinsella, 1988). It is observed that β-Lg undergoes heat denaturation and self-aggregation involving –SH/–SS– exchange reactions before forming a complex with κ-C (Sawyer, 1969; Dziuba, 1979; Haque et al., 1987). Since sucrose is known to inhibit –SH/–SS– exchange reactions and thermal coagulation of β-Lg during heat treatment (Yamauchi, 1961; Garrett et al., 1988), it probably prevented, to some extent, the association of β-Lg with κ-C, thus facilitating chymosin hydrolysis. Although retinol is known to form stable complexes with β-Lg (Hemely et al., 1979; Fugate and Song, 1980), it did not obviously affect the interaction of β-Lg with κ-C (Figure 1). Sugimoto et al. (1974) reported that ANS binds to α_s-, β-, and κ-caseins and prevents the association between α_s-, β-, and κ-casein. It is not certain whether the binding of ANS to casein components had any effect on the hydrophobic interaction

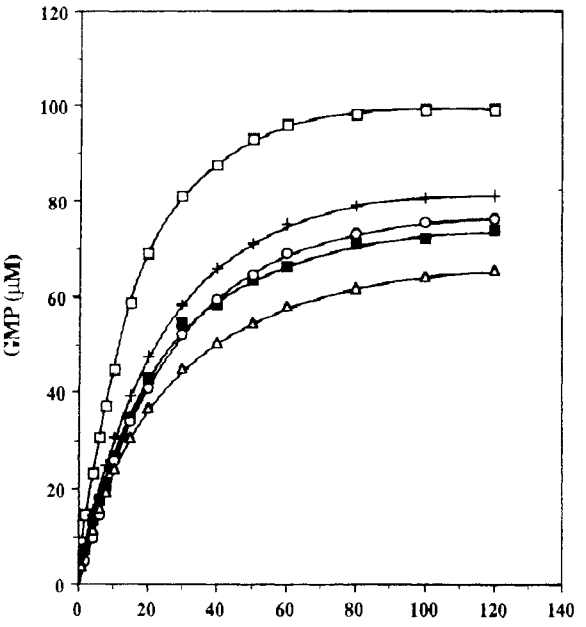

Figure 1. Effect of sucrose, retinol, and ANS on the heat-induced interaction of β-lactoglobulin with κ-casein in micelles as shown by kinetics of chymosin hydrolysis of κ-casein (heating at 85 °C for 10 min; chymosin, 5 μg/10 mL of substrate): casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated (■); casein micelles + β-lactoglobulin, heated in the presence of sucrose (+), retinol (○), and ANS (△).

Table I. Initial Velocity (V_i) of and Total Amount of Glycomacropeptide (GMP) Released by the Chymosin Hydrolysis of Casein Micelles and β-Lactoglobulin Solutions Heated (85 °C for 10 min) in the Presence of Sucrose, Retinol, and ANS

	V_i		GMP[a]	
	μM/s	%	μM	%
casein micelles + β-lactoglobulin, unheated	8.17×10^{-2}	100.00	99.00	100.00
casein micelles + β-lactoglobulin, heated	5.17×10^{-2}	63.28	73.80	74.54
casein micelles + β-lactoglobulin, heated in the presence of				
sucrose (6.67% w/v)	6.00×10^{-2}	73.44	81.00	81.82
retinol (7.2×10^{-4} M)	4.33×10^{-2}	53.00	76.20	76.97
ANS (1.04×10^{-3} M)	4.33×10^{-2}	53.00	65.40	66.06

[a] At the end of 120-min hydrolysis.

between β-Lg and κ-C, but it inhibited enzymic reaction.

Effect of Sodium Sulfite and SDS. These were studied by heating a mixture of casein micelles and β-Lg in the presence of 5.0×10^{-3} M sodium sulfite or 5.2×10^{-3} M SDS and determining the kinetics of chymosin hydrolysis (Figure 2), V_i and GMP released (Table II). The data show that sodium sulfite improved chymosin hydrolysis to some extent. It did not affect V_i but increased the GMP released by 15%, indicating that sodium sulfite caused a partial inhibition of complex formation between β-Lg and κ-C.

Zittle et al. (1962) reported that complex formation could be prevented by heating a mixture of β-Lg and κ-C in the presence of β-mercaptoethanol, a reducing agent, and *N*-ethylmaleimide, a thiol-blocking agent. Sodium sulfite has been used to cleave disulfide bonds of β-Lg and other proteins by oxidative sulfitolysis (Swan, 1957; Kella and Kinsella, 1985; Mohan Reddy et al., 1989). Therefore, addition of sodium sulfite to the reaction mixture

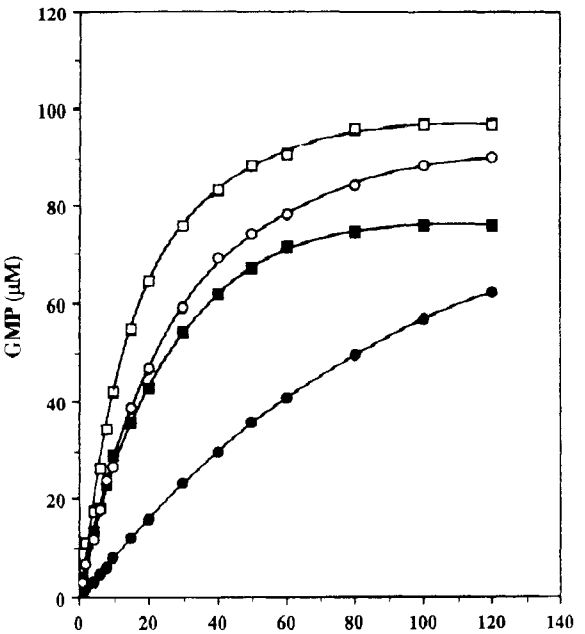

Figure 2. Effect of SDS and sodium sulfite on the heat-induced interaction of β-lactoglobulin with κ-casein in micelles as shown by kinetics of chymosin hydrolysis of κ-casein (heating at 85 °C for 10 min; chymosin, 5 μg/10 mL of substrate): casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated (■); casein micelles + β-lactoglobulin, heated in the presence of SDS (●) and sodium sulfite (○).

Table II. Initial Velocity (V_i) of and Total Amount of Glycomacropeptide (GMP) Released by the Chymosin Hydrolysis of Casein Micelles and β-Lactoglobulin Solutions Heated (85 °C for 10 min) in the Presence of SDS, Sodium Sulfite, Lysozyme, and Ovalbumin

	V_i		GMP[a]	
	μm/s	%	μM	%
casein micelles + β-lactoglobulin, unheated	8.00×10^{-2}	100.00	96.60	100.00
casein micelles + β-lactoglobulin, heated	5.17×10^{-2}	64.62	75.60	78.26
casein micelles + β-lactoglobulin, heated in the presence of				
SDS (5.2×10^{-3} M)	1.33×10^{-2}	16.62	62.10	64.28
sodium sulfate (5.0×10^{-3} M)	5.08×10^{-2}	63.50	90.00	93.16
lysozyme (0.4% w/v)	8.17×10^{-2}	102.12	92.00	95.24
ovalbumin (0.5% w/v)	4.33×10^{-2}	54.12	70.59	73.07

[a] At the end of 120-min hydrolysis.

before heating might have prevented the complex formation by cleaving disulfide bonds and simultaneously S-sulfonating the free -SH groups (Swan, 1957; Kella and Kinsella, 1985). This supports the earlier observations (Sawyer, 1969) that intermolecular -SS- bonds play a role in complex formation between β-Lg and κ-C.

SDS clearly had a detrimental effect on chymosin hydrolysis (Figure 2). It further decreased the V_i and GMP released (Table II) compared to the mixture of casein micelles and β-Lg heated without SDS. Steinhardt and Reynolds (1969) reported that binding of anionic detergent SDS at specific sites stabilizes protein structure. β-Lg is reported to have specific high-affinity binding sites for amphiphiles (Helenius and Simons, 1975), and it binds two molecules of SDS per mole (McMeekin et al., 1949). Hegg (1980) reported that binding of two molecules of SDS per β-Lg molecule increased its thermal stability.

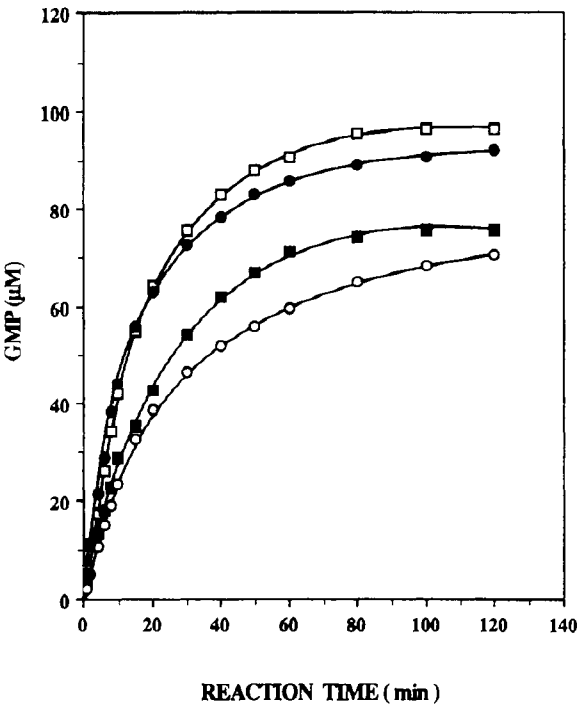

Figure 3. Effect of lysozyme and ovalbumin on the heat-induced interaction of β-lactoglobulin with κ-casein in micelles as shown by kinetics of chymosin hydrolysis of κ-casein (heating at 85 °C for 10 min; chymosin, 5 μg/10 mL of substrate): casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated (■); casein micelles + β-lactoglobulin, heated in the presence of lysozyme (●) and ovalbumin (○).

SDS was also shown to interact hydrophobically with the caseins (Cheeseman and Jeffcoat, 1970; Cheeseman and Knight, 1970). In view of the above, SDS is unlikely to promote the complex formation between β-Lg and κ-C. On the other hand, binding of negatively charged SDS (net charge of −1 per mol) to casein micelles as well as β-Lg might have caused a further increase in net negative charge of casein micelle/β-Lg complex, resulting in an increased electrostatic repulsion between the enzyme and its substrate. This is a possible explanation for drastic inhibition of chymosin hydrolysis of the κ-C on casein micelles and β-Lg mixture heated in the presence of SDS and also supports the idea that inhibition of chymosin hydrolysis as a result of the interaction of β-Lg with κ-C may involve electrostatic repulsion between the enzyme and substrate, resulting from the increased net negative charge of casein micelle/β-Lg complex.

Effect of Ovalbumin and Lysozyme. It has been shown that binding of lysozyme and ovalbumin to native casein micelles markedly decreased rennet clotting time (Green and Marshall, 1977, 1979; Marshall and Green, 1980). Therefore, it was of interest to ascertain whether the addition of these proteins to the reaction mixture prior to heating affected chymosin hydrolysis. This was checked by adding ovalbumin (0.5% w/v) and lysozyme (0.4% w/v) to casein micelles and β-Lg mixture before heating and following the kinetics of chymosin hydrolysis. The results of time course of chymosin hydrolysis (Figure 3) and V_i and GMP released (Table II) indicate that inhibition of chymosin hydrolysis as a result of interaction of β-Lg with κ-C was almost completely reversed by lysozyme. V_i (8.17×10^{-2} μM/s) and GMP released (92 μM) in the presence of lysozyme were almost equal to the values obtained for an unheated mixture of casein micelles and β-Lg (8.00×10^{-2} μM/s and 96.6 μM, respectively). It is not certain whether lysozyme prevented the complex formation between β-Lg and micellar κ-casein. At the pH of the reaction mixture (pH 6.8) the basic protein, lysozyme (p*I* 10.7) is positively charged and can interact electrostatically with negatively charged proteins. In the casein micelle several areas are potentially available for the binding of cationic materials. The calcium–phosphate–citrate complex is negatively charged (Pyne and McGann, 1960), and cationic materials could be expected to bind to it (Ho and Waugh, 1965a,b). The carboxyl residues in the caseins, which together with the calcium–phosphate–citrate complex give the micelle its overall negative charge (Green and Crutchfield, 1971), bind divalent metal cations (Dickson and Perkins, 1971). Therefore, the positively charged lysozyme could be expected to bind to these residues and neutralize the overall negative charge of the casein micelle/β-Lg complex. Similarly, it may affect β-Lg (p*I* 5.3), which is negatively charged at pH 6.8. It is postulated that heat-induced interaction of β-Lg with κ-C may lead to an increase in net negative charge of the micelles, resulting in electrostatic repulsion between the enzyme and its substrate (Mohan Reddy and Kinsella, 1989). The presence of lysozyme in the mixture of casein micelles and β-Lg before heating may overcome this effect and facilitate enzyme–substrate complex formation, in a manner similar to that occurring in native micelles. It may also affect the secondary (ionic) interactions between β-Lg and κ-C. This seems to be the possible mechanism of action of lysozyme in overcoming or preventing the inhibition of chymosin hydrolysis of κ-C as a result of its interaction with β-Lg.

On the other hand, the presence of ovalbumin during heating of the mixture of casein micelles and β-Lg decreased the rate of chymosin hydrolysis (Figure 3). The V_i and amount of GMP released were further decreased compared to the reaction mixture heated without ovalbumin (Table II). This may indicate that ovalbumin and β-Lg have possibly acted additively in inhibiting the chymosin hydrolysis of κ-C. Sato et al. (1977) reported that ovalbumin (p*I* 4.6) when heated binds to κ-C by electrostatic interactions and to β-casein by hydrophobic bonds but did not involve interaction between the −SH groups of ovalbumin and κ-C. Conceivably the interaction of ovalbumin with κ-C also inhibits chymosin hydrolysis by increased electrostatic repulsion between the enzyme and substrate, or it may reflect steric hindrance.

Effect of Cetyltrimethylammonium Bromide and Cetylpyridinium Chloride. Inhibition of chymosin hydrolysis of κ-C as a result of its interaction with β-Lg was completely prevented when a mixture of casein micelles and β-Lg was heated in the presence of lysozyme, a positively charged protein. In the presence of a negatively charged detergent, SDS, however, chymosin hydrolysis was further inhibited. It was felt that binding of SDS to casein micelles as well as β-Lg might have caused a further increase in net negative charge of the casein micelle/β-Lg complex, resulting in increased electrostatic repulsion between the enzyme and substrate. On the other hand, binding of lysozyme to casein micelles may have neutralized the overall negative charge of the casein micelle/β-Lg complex, thereby facilitating the enzyme–substrate complex formation. This hypothesis was further checked by using positively charged detergents, CTAB and CPC. The detergents carry a net charge of +1 per mole and have been reported to bind casein micelles and markedly reduce the rennet clotting time (Pearce, 1976; Green and Marshall, 1979; Marshall and Green, 1980). Since β-Lg carries a net charge of −10 per mole at pH 6.6 (Basch and Timasheff, 1967), the detergents are also expected to bind to it. Therefore, the addition of CTAB and CPC to the mixture of casein micelles

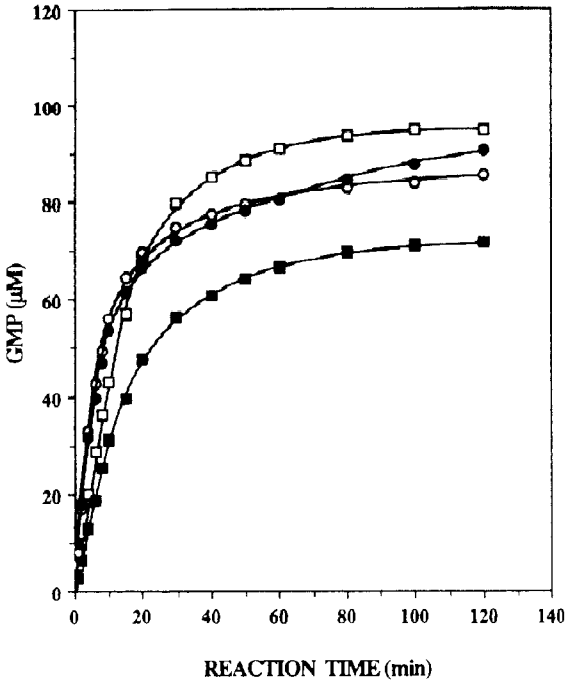

Figure 4. Effect of cetyltrimethylammonium bromide and cetylpyridinium chloride on the heat-induced interaction of β-lactoglobulin and κ-casein in micelles as shown by kinetics of chymosin hydrolysis of κ-casein (heated at 85 °C for 10 min; chymosin, 5 μg/10 mL of substrate): casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated (■); casein micelles + β-lactoglobulin, heated in the presence of CTAB (●) and CPC (○).

Table III. Initial Velocity (V_i) of and Total Amount of Glycomacropeptide (GMP) Released by the Chymosin Hydrolysis of Casein Micelles and β-Lactoglobulin Heated (85 °C for 10 min) in the Presence of Cetyltrimethylammonium Bromide (CTAB) and Cetylpyridinium Chloride (CPC)

	V_i		GMP[a]	
	μm/s	%	μM	%
casein micelles + β-lactoglobulin, unheated	8.00×10^{-2}	100.00	94.84	100.00
casein micelles + β-lactoglobulin, heated	5.33×10^{-2}	66.62	71.50	75.39
casein micelles + β-lactoglobulin, heated in the presence of				
CTAB (2.6×10^{-3} M)	12.92×10^{-2}	161.50	85.50	90.15
CPC (2.6×10^{-3} M)	12.83×10^{-3}	160.37	90.45	95.37

[a] At the end of 120-min hydrolysis.

and β-Lg before heating was expected to improve the chymosin hydrolysis of κ-C.

The effects of heating casein micelles and β-Lg mixture in the presence of CTAB (2.6×10^{-3} M) or CPC (2.6×10^{-3} M) on the chymosin hydrolysis of κ-C (Figure 4; Table III) indicate that inhibition of enzyme hydrolysis as a result of interaction of β-Lg with κ-C was prevented by these detergents. The values of V_i in the presence of CTAB and CPC (12.92×10^{-2} and 12.83×10^{-2} μM/s, respectively) were higher than the value obtained for unheated casein micelles and β-Lg mixture (8.00×10^{-2} μM/s). The release of GMP was increased by about 20% in the presence of detergents. The results confirm the mechanism of action of lysozyme in preventing the inhibition of chymosin hydrolysis of κ-C as a result of its interaction with β-Lg.

Effect of the Addition of Lysozyme, CTAB, and CPC to the Heated Mixture of Casein Micelles and β-Lg on Chymosin Hydrolysis. If the above mechanism of action of lysozyme, CTAB, and CPC is valid, the addition of these materials to the heated casein micelles and β-Lg mixture prior to renneting should also prevent the inhibition of chymosin hydrolysis of κ-C as a result of its interaction with β-Lg. This was checked by adding lysozyme (0.4% w/v), CTAB (2.6×10^{-3} M), and CPC (2.6×10^{-3} M) to the heated and cooled mixture of casein micelles and β-Lg following the chymosin hydrolysis. The results indicate that all the three additives improved chymosin hydrolysis. The values of V_i for unheated and heated reaction mixtures were 13.67×10^{-2} and 6.50×10^{-2} μM/s, respectively. The values for heated mixtures with added lysozyme, CTAB, and CPC were 13.60×10^{-2}, 13.50×10^{-2}, and 13.60×10^{-2} μM/s, respectively. The V_i values for unheated and heated samples were high because of the higher enzyme concentration (5.5 μg/10 mL substrate) used in this experiment. The results further confirm that the addition of positively charged lysozyme and detergents to casein micelles and β-Lg mixture before or after heating prevents the inhibition of chymosin hydrolysis of κ-C, conceivably by neutralizing the overall negative charge of the casein micelle/β-Lg complex.

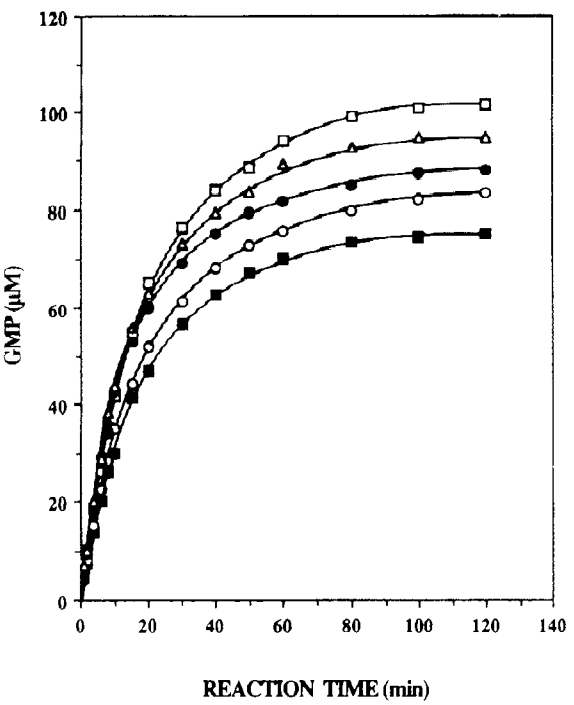

Figure 5. Effect of lysozyme concentration on the heat-induced interaction of β-lactoglobulin and κ-casein in micelles as shown by kinetics of chymosin hydrolysis of κ-casein (heating at 85 °C for 10 min; chymosin, 5 μg/10 L of substrate): casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated (■); casein micelles + β-lactoglobulin, heated in the presence of 0.1% lysozyme (○), 0.2% lysozyme (●), and 0.4% lysozyme (△).

Effect of Lysozyme Concentration on Casein Micelles and β-Lg Mixture. In order to determine the optimum concentration of lysozyme required to prevent the inhibition of chymosin hydrolysis, a mixture of casein micelles and β-Lg was heated in the presence of different concentrations of lysozyme (0.1–0.4%). The results (Figure 5; Table IV) indicate that the rate of chymosin hydrolysis increased with an increase in lysozyme concentration from 0.1 to 0.4%. As observed in Figure 3, the inhibition of chymosin hydrolysis as a result of interaction of β-Lg with κ-C was almost completely reversed at 0.4% lysozyme. This may be the optimum concentra-

Table IV. Initial Velocity (V_i) of and Total Amount of Glycomacropeptide (GMP) Released by the Chymosin Hydrolysis of Casein Micelles and β-Lactoglobulin Solutions Heated (85 °C for 10 min) in the Presence of Different Concentrations of Lysozyme

	V_i		GMP[a]	
	μm/s	%	μM	%
casein micelles + β-lactoglobulin, unheated	7.92×10^{-2}	100.00	101.40	100.00
casein micelles + β-lactoglobulin, heated	5.25×10^{-2}	66.28	75.00	73.96
casein micelles + β-lactoglobulin, heated in the presence of				
0.1% lysozyme	6.42×10^{-2}	81.06	83.40	82.25
0.2% lysozyme	7.67×10^{-2}	96.84	88.20	86.98
0.4% lysozyme	8.17×10^{-2}	103.16	94.80	93.49

[a] At the end of 120-min hydrolysis.

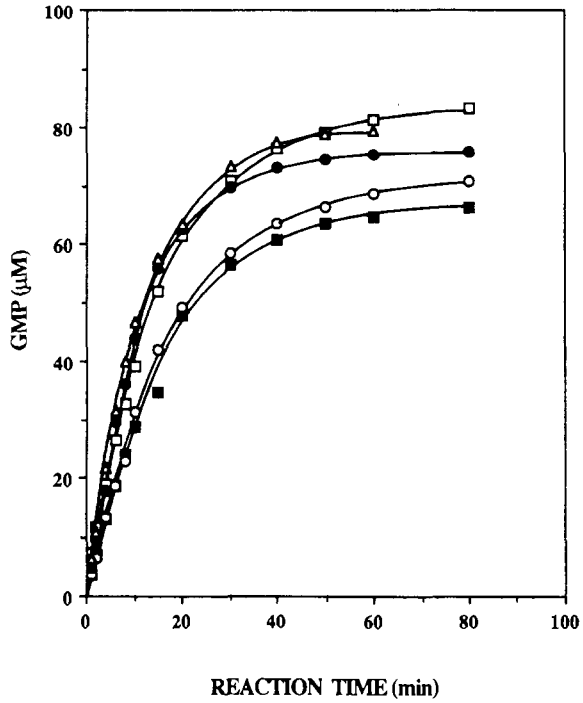

Figure 6. Effect of lysozyme concentration on the heat-induced interaction of β-lactoglobulin and κ-casein in skim milk as shown by kinetics of chymosin hydrolysis of κ-casein (heating at 85 °C for 10 min; chymosin, 5 μg/10 mL of substrate): skim milk, unheated (□); skim milk, heated (■); skim milk, heated in the presence of 0.1% lysozyme (○), 0.2% lysozyme (●), and 0.4% lysozyme (△).

tion of lysozyme required to neutralize the negative charge contributed by β-Lg to casein micelles as a result of its interaction with micellar κ-C. Lysozyme may also bind β-Lg electrostatically prior to heating and may prevent the secondary (ionic) interactions of β-Lg with positive cluster around the Phe^{105}–Met^{106} bond of κ-C during heating.

Effect of Lysozyme Concentration on Skim Milk. Studies were carried out with skim milk in order to test the effects of lysozyme in preventing the inhibition of chymosin hydrolysis as a result of the interaction of β-Lg with κ-C in model systems. Skim milk was heated at 85 °C for 10 min with different concentrations of lysozyme, and the kinetics of chymosin hydrolysis was followed after cooling. The results (Figure 6; Table V) indicate that the rate of chymosin hydrolysis increased with an increase in lysozyme concentration and at 0.4% lysozyme the inhibition of enzyme hydrolysis was completely reversed. V_i

Table V. Initial Velocity (V_i) of and Total Amount of Glycomacropeptide (GMP) Released by the Chymosin Hydrolysis of Skim Milk Heated (85 °C for 10 min) in the Presence of Different Concentrations of Lysozyme

	V_i		GMP[a]		rennet clotting time,[b] min
	μm/s	%	μM	%	
skim milk, unheated	8.08×10^{-2}	100.00	81.31	100.00	60
skim milk, heated	5.42×10^{-2}	67.08	64.55	73.38	no clotting
skim milk, heated in the presence of					
0.1% lysozyme	5.50×10^{-2}	68.07	68.54	84.29	no clotting
0.2% lysozyme	8.33×10^{-2}	103.03	75.10	92.36	no clotting
0.4% lysozyme	9.17×10^{-2}	113.49	79.20	97.40	60

[a] At the end of 60-min hydrolysis. [b] Visual clotting time followed up to 120-min hydrolysis.

values at 0.2 and 0.4% lysozyme (8.33×10^{-2} and 9.17×10^{-2} μM/s, respectively) were higher than that obtained for unheated milk (8.08×10^{-2} μM/s). GMP values for unheated milk and the milk heated with 0.4% lysozyme were almost the same. Visual clotting times for unheated milk and milk heated with 0.4% lysozyme were the same, i.e., 60 min, respectively. Thus, lysozyme at 0.4% concentration could completely prevent the inhibition of chymosin hydrolysis of heated milk as a result of the interaction of β-Lg with κ-C and restore the rennet clotting time to equal that of unheated milk.

In summary, the inhibition of chymosin hydrolysis of κ-C as a result of its interaction with β-Lg was prevented by the addition of positively charged protein, lysozyme, and cationic detergents, CTAB and CPC. The addition of sucrose and sodium sulfite also improved chymosin hydrolysis to some extent. Addition of anionic detergent, SDS, negatively charged protein, ovalbumin, and hydrophobic ligand, ANS, further inhibited chymosin hydrolysis. Retinol, which is known to bind β-Lg with high affinity, did not influence chymosin hydrolysis. Lysozyme, CTAB, and CPC probably acted by neutralizing the negative charge of casein micelle/β-Lg complex, thereby facilitating enzyme–substrate complex formation. SDS and ovalbumin probably increased further the net negative charge of the casein micelle/β-Lg complex, resulting in increased electrostatic repulsion between the enzyme and its substrate.

An optimum concentration of lysozyme required for the inhibition of chymosin hydrolysis of κ-C in heated casein micelles and β-Lg mixture was 0.4%. Lysozyme at 0.4% concentration also prevented the inhibition of chymosin hydrolysis of heated milk and restored the rennet clotting time to equal that of unheated milk. These findings may afford an approach for minimizing the formation of β-Lg/κ-c complexes during the thermal treatments involved in preparation of milk powders and allow these to be used in conventional cheesemaking.

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