Overnell, J. Digestive enzymes of the pyloric caeca and of their associated mesentry in the cod. *Comp. Biochem. Physiol.* **1973**, *46B*, 519-531.
Petra, P. H. Bovine procarboxypeptidase and carboxypepti- dase A. *Methods Enzymol.* **1970**, *19*.
Pfleiderer, G. Particle-bound aminopeptidase from pig kidney. *Methods Enzymol.* **1970**, *19*.
Prahl, J. W.; Neurath, H. Pancreatic enzymes of the spiny dogfish I. Cationic chymotrypsinogen and chymotrypsin. *Biochemistry* **1966**, *5*, 2131-2145.
Raa, J.; Gildberg, A. Autolysis and proteolytic activity of cod viscera. *J. Food Technol.* **1976**, *11*, 619-628.
Raa, J.; Gildberg, A.; Strom, T. Silage production-Theory and practice. In *Upgrading wastes for feeds and food*; Ledward, D. A., Taylor, A. J., Lawrie, R. A., Eds.; Butterworths: London, 1983.
Rattagoal, P. P.; Wongchinda, N.; Swachathamwongratana, S. Studies on the nutritive value of fish silage for broiler chickens. In *Proceedings of the IPFC Workshop on fish silage production and its use*; FAO: Jakarta, Indonesia, 1979; pp 48-54.
Reece, P. Control and reduction of free fatty acid concentration in oil recovered from fish silage prepared from sprat. *J. Sci. Food Agric.* **1980**, *31*, 147-155.
Reece, P. Recovery of proteases from fish waste. *Process Biochem.* **1988**, *23*, 62-66.
Rosen, H. A modified ninhydrin colorimetric analysis for aminoacids. *Arch. Biochem. Biophys.* **1957**, *671*, 10-15.
Sakai-Suzuki, J.; Tobe, M.; Tsuchiya, T.; Matsumoto, J. J. Purification and characterization of acid cysteine proteinase from squid mantle muscle. *Comp. Biochem. Physiol.* **1986**, *85B*, 887-893.
Shin, D. H.; Zall, R. R. Purification and identification of a trypsin-like enzyme from the pyloric caeca of cod. *Process Biochem.* **1986**, *21*, 11-15.
Stone, F. S.; Hardy, R. W. Nutritional value of acid stabilized silage and liquefied fish protein. *J. Sci. Food Agric.* **1986**, *37*, 797-803.
Stone, F. S.; Hardy, R. W.; Spineldin, J. Autolysis of phytic acid and protein in Canola meal, wheat bran and fish silage blends. *J. Sci. Food Agric.* **1984**, *35*, 513-519.
Tarky, W.; Agarwala, G. P.; Pigot, G. M. Protein hydrolyzate from fish waste. *J. Food Sci.* **1973**, *38*, 917-918.
Tatterson, I. N.; Windsor, M. L. Fish silage. *J. Sci. Food Agric.* **1974**, *25*, 369-379.
Twining, S. S.; Alexander, P. A.; Huibregtse, K.; Glick, D. A pepsinogen from rainbow trout. *Comp. Biochem. Physiol.* **1983**, *75B*, 109-112.
VoVan, T.; Kusakabe, I.; Murakami, K. Properties of two aminopeptidases from sardines. *Agric. Biol. Chem.* **1983**, *47*, 2453-2454.
Walsh, K. A.; Wilcox, P. E. Serine proteases. *Methods Enzymol.* **1970**, *19*.
Wilcox, P. E. Chymotrypsinogens-Chymotrypsins. *Methods Enzymol.* **1970**, *19*.

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Interaction of β-Lactoglobulin with κ-Casein in Micelles As Assessed by Chymosin Hydrolysis: Effect of Temperature, Heating Time, β-Lactoglobulin Concentration, and pH

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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. The rate of enzymic reaction was determined on the basis of the release of glycomacropeptide (GMP) soluble in 8% TCA. Heating of β-Lg with κ-C and with casein micelles in Jenness-Koops buffer at pH 6.8 inhibited chymosin hydrolysis, resulting in decreased initial velocity (V_i) and GMP release. Heating casein micelles alone did not affect chymosin hydrolysis. V_i and the release of GMP decreased steadily with increasing temperature of heating from 60 °C and reached a maximum at 85 °C. The decreases in V_i and GMP release at 85 °C were 37 and 26%, respectively. Inhibition reached a maximum after heating for 10 min at 85 °C, with 38% decrease in V_i and 28% decrease in GMP. A heating time of 1 min at 85 °C caused a 9% decrease in V_i and 15% decrease in GMP release. Decrease in V_i and the release of GMP was proportional to the concentration of β-Lg (0.05–0.50%) added to casein micelles. Heating of casein micelles and β-Lg at pH 6.0 or 7.5 did not improve chymosin hydrolysis, whereas lowering to pH 5.8 and readjusting to pH 6.8 before chymosin addition improved chymosin hydrolysis.

The heat-induced interaction of β-lactoglobulin (β-Lg) and κ-casein (κ-C) has been extensively studied (Sawyer, 1969; Smits and van Brouwershaven, 1980; Euber and Brunner, 1982; Doi et al., 1981, 1983; Haque et al., 1987; Haque and Kinsella, 1988). Using ^{3}H-labeled β-Lg, Smits and van Brouwershaven (1980) presented evidence of association of casein micelles and β-Lg. The existence of a β-Lg and κ-C complex in heated milk has been reported (Snoeren and van der Spek, 1977; Elfagm and Whee-

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lock, 1977; Parnell-Clunies et al., 1988). It is clearly established that the formation of intermolecular –SS– bonds between β-Lg and κ-C as well as hydrophobic interactions is involved in this interaction (Sawyer, 1969; Dziuba, 1979; Smits and van Brouwershaven, 1980; Doi et al., 1983; Haque and Kinsella, 1988). It has been suggested that this complex formation is responsible for the increased clotting time and the reduction in total amount of peptides releasedby chymosin when milk is heated (Sawyer, 1969; Hindle and Wheelock, 1970; Wilson and Wheelock, 1972).

Several investigators have studied the renneting properties of heated milk (Hindle and Wheelock, 1970; Wilson and Wheelock, 1972; Wilson et al., 1974; Domicz and Dziuba, 1975; Marshall, 1986; van Hooydonk et al., 1987; Singh et al., 1988). Because of the complex nature of milk, the results of such studies are better interpreted when carried out in model systems containing casein micelles and β-Lg. Wheelock and Kirk (1974) and Shalabi and Wheelock (1977) studied the effect of hydrolysis of κ-C by chymosin following heating of an aqueous mixture of casein micelles and β-Lg at 90 °C for 1 h (under reflux) on the primary phase of chymosin action by estimating the total peptides (as ppm, N) soluble in 2% trichloroacetic acid (TCA). However, native β-Lg interferes with the estimation of 2% TCA soluble peptides hydrolyzed by chymosin. Moreover, under the drastic heating conditions (90 °C for 1 h) used, loss of –SH and –SS– sulfur in β-Lg A has been reported (Watanabe and Klostermeyer, 1976). Degradation of –SH and –SS– residues may result in decreased interaction of β-Lg with κ-C (Long et al., 1963). In view of the above, a study of the interaction of β-Lg with κ-C on intact micelles under moderate heating conditions using an improved method to determine TCA-soluble glycomacropeptide (GMP) (van Hooydonk and Oleiman, 1982) was undertaken to ascertain whether this reaction could be controlled.

In this investigation, a systematic study of the effects of heating temperature, heating time, β-Lg concentration, and pH on the interaction of β-Lg with κ-C on intact micelles was studied by following a complete time course of chymosin hydrolysis. The rate of enzymatic reaction was determined on the basis of the release of GMP soluble in 8% TCA. Initial velocities of the enzymatic reaction under different conditions of the interaction were calculated. The results are discussed in light of reported changes in conformation and –SH and –SS– contents of β-Lg under different conditions of temperature and pH. Also, the possible mechanism of inhibition of chymosin hydrolysis of κ-C as a result of its interaction with β-Lg is discussed.

MATERIALS AND METHODS

Materials. Fresh skim milk (Holstein) was obtained from the Cornell Dairy Plant. Chymosin (activity approximately 60 units/mg of protein) and fluorescamine were purchased from Sigma Chemical Co., St. Louis, MO. Spectral-grade ACS-certified acetone was obtained from Fisher Scientific. 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 by gel filtration on Sephadex G-100 in 0.1 M phosphate buffer, pH 6.8, containing 0.4 M NaCl, dialyzed against distilled water, lyophilized, and stored at 4 °C under desiccation.

κ-Casein was isolated from freshly prepared acid–casein by the procedure of Zittle and Custer (1963) as modified by Doi et al. (1979). It was further purified by a linear NaCl elution from DEAE–Sephacel, in 3 M urea/0.02 M imidazole buffer, pH 7.0 containing 0.1% 2-mercaptoethanol (Doi et al., 1979). Fractions P2–P6 (Doi et al., 1979) were pooled and dialyzed against deionized water containing 0.1% 2-mercaptoethanol at 4 °C, lyophilized, and stored at 4 °C under desiccation.

Glycomacropeptide was prepared from purified κ-C by treating with chymosin as described by Ono et al. (1987). After addition of chymosin to the κ-C solution containing 0.07 M NaCl, the mixture was incubated at 35 °C for 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 a Sorval OTD-65B ultracentrifuge (Du Pont). The casein pellet was redispersed in simulated milk salt 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. 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 and κ-C were dissolved in modified milk salt buffer, pH 6.8, and centrifuged at 20000*g* for 30 min. The concentrations of β-Lg and κ-C were determined spectrophotometrically at 280 nm by using $E^{1\%}_{1cm}$ values of 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 a protein value (Karman and van Boekel, 1986).

Preparation of Reaction Mixtures. To 7.5 mL of casein micelle solution (approximately 30 mg/mL) was added 1.0 mL of 3% β-Lg solution in modified milk salt buffer, pH 6.8, and the volume made to 10.0 mL in the same buffer. The final concentrations of the total casein (approximately 2.25%) and β-Lg (0.3%) in the reaction mixture were approximately the same as in milk, and thus a 1:1 ratio of β-Lg to κ-C was maintained. A model system containing a purified κ-C and β-Lg mixture at a concentration of 0.3% each was also used. In some experiments raw skim milk was also used.

Heat Treatments. Aliquots (10 mL) of the reaction mixture were heated in screw-cap tubes (25 mL) at specific temperatures in a thermostatically controlled water bath. Each sample was preheated for 3–4 min to attain the desired temperatures and held at the specified temperature for various times. After heating, the tubes were cooled to 15 °C in water and stored overnight at 5 °C.

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 (van Hooydonk and Oleiman, 1982). Aliquots (0.5 mL) were withdrawn at regular intervals from the reaction mixture after the addition of enzyme, 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%. TCA immediately inactivates chymosin and precipitates the casein and the whey proteins (van Hooydonk and Oleiman, 1982). An aliquot devoid of the enzyme, which was treated in the same way with TCA, was used as a blank. After 1-h storage at 30 °C the precipitated samples were filtered (Whatman 42) and the filtrates analyzed for GMP by HPLC (van Hooydonk and Oleiman, 1982) and by the fluorescence method (Beeby, 1980).

Effect of pH. pH of the reaction mixture was adjusted to 6.0 with 5 N HCl or 7.5 with 5 N KOH, and the mixture was heated at 85 °C for 10 min as described above. Before chymosin addition, the samples were brought to room temperature and the pH was readjusted to 6.8. Alternately, the pH of the heated and cooled reaction mixture was dropped to 5.8 and immediately raised to pH 6.8 before chymosin addition.

Estimation of GMP. *HPLC Method.* The analytical procedure was essentially that described by van Hooydonk and Oleiman (1982), with some modifications. The analyses were performed by using a system consisting of a Waters M-45 solvent delivery system, a Zorbex GF-250 gel filtration column (2.5 cm

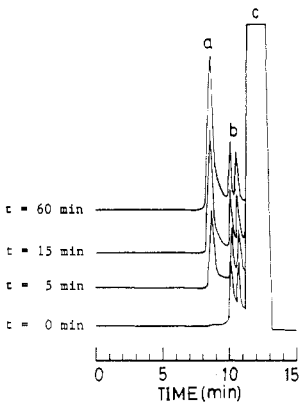

Figure 1. Chromatogram showing HPLC separation of glycomacropeptide after 0, 5, 15, and 60 min of chymosin hydrolysis. Key: (a) glycomacropeptide; (b) nonprotein nitrogen; (c) trichloroacetic acid. Conditions: flow rate, 1 mL/min; detection, 220 nm; 0.1 AUFS; injection volume, 5 μL.

× 0.94 cm (i.d.); Du Pont), a Waters Model 450 variable-wavelength UV detector, and a Hewlett-Packard 3390A integrator.

The eluant consisted of a solution of 1.74 g of K_2HPO_4, 12.37 g of KH_2PO_4, and 21.41 g of Na_2SO_4 in 1000 mL of double-distilled water (pH 5.6). The eluant was filtered through 0.45-μm Millipore filter and degassed under vacuum with a sonicator.

For each analysis, 5 μL of the sample, i.e., the supernatant after TCA precipitation, was injected manually. The flow rate was set at 1.0 mL/min and the UV detector operated at 220 nm and 0.1 AUFS.

Fluorescence Method. The GMP was estimated fluorometrically with fluorescamine as described by Beeby (1980). An aliquot (100 μL) of the blank as well as sample was added to 3.0 mL of a sodium tetrafluoroborate solution (0.2 M), which had been 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, and after 15 min the fluorescence was measured at 480 nm (excitation 390 nm) with a Perkin-Elmer 650-40 fluorescence spectrophotometer. All assays were performed at 25 °C. A standard graph of GMP (μM) vs fluorescence at 480 nm was constructed with use of purified GMP. The fluorescence readings were converted into GMP with the standard graph and expressed in micromoles. A molecular weight of 8000 is used for GMP.

The HPLC separation of GMP, NPN, and TCA together with the increase in peak height of GMP with an increase in time of hydrolysis is shown in Figure 1. The NPN peak height did not vary with the time of hydrolysis. There was no indication of other TCA-soluble degradation products with molecular sizes differing from GMP being produced during hydrolysis. Since each run takes about 15 min to produce a complete chromatogram, it was time-consuming to analyze a large number of samples by HPLC. Hence, GMP was routinely estimated by the fluorescence method (Beeby, 1980). The fluorescence value of the blank (zero-time peak), which is contributed solely by NPN (Figure 1), was always subtracted from the values obtained after the addition of chymosin. Thus, the fluorescence values obtained for samples were proportional to the release of GMP with time of hydrolysis.

Calculation of Initial Velocity. V_i of chymosin hydrolysis was calculated as follows. A line tangent to the initial part of the experimental curve possibly through the origin was drawn. V_i was then calculated from the slope of the line and expressed in micromoles per second.

RESULTS AND DISCUSSION

Interaction of β-Lg with κ-C in heated solutions of κ-C and β-Lg and of casein micelles and β-Lg and in heated skim milk as assessed by chymosin hydrolysis is shown in Figure 2. The initial velocities of chymosin hydrolysis and final amount of glycomacropeptide (GMP) released are given in Table I. The hydrolysis of casein micelles and β-Lg system was followed up to 120 min, whereas, owing to the initiation of clotting process after 80 min, the hydrolysis of κ-C and β-Lg and of skim milk systems was followed up to 80 min only. Hence, the final GMP values in the latter case are given at the end of 80-min hydrolysis (Table I).

Although the initial concentration of κ-C was approximately the same in all cases, the V_i was lower in the case of casein micelle as well as milk substrate. This suggests that the κ-C molecules were more difficult to attack or were less accessible when present in the native micelles. It is clear that heating affected the kinetics of chymosin hydrolysis in all cases and decreased the final GMP released by 20–25%. There was a significant reduction of V_i of heated casein micelles and β-Lg and of heated skim milk systems compared to heated κ-C and β-Lg system. This suggests that the conformational disposition of the Phe^{105}–Met^{106} bond of κ-C on intact micelle surface after complex formation with β-Lg is somewhat different and probably not easily accessible to the enzyme. Contrary to the earlier observation of Wheelock and Kirk (1974), heating of casein micelles alone did not have any effect on chymosin hydrolysis (Figure 2B) and in fact resulted in a slight increase in V_i and final GMP released. This may reflect some heat-induced dissociation of κ-C from casein micelles (Hindle and Wheelock, 1970; Marshall, 1986; van Hooydonk et al., 1987).

In the course of experiments, we found that increasing the sodium citrate content (from 6.0 to 7.2 mM) in simulated milk salt buffer (i.e., modified milk salt buffer) prolonged the rennet clotting time of unheated and heated casein micelles. Therefore, the use of modified milk salt buffer in this study allowed us to follow a complete time course of chymosin hydrolysis for 120 min.

Effect of Temperature. The kinetics of chymosin hydrolysis of κ-C on casein micelles following heating with β-Lg at different temperatures (60–90 °C) for 30 min are summarized in Figure 3A. Figure 3B gives a plot of V_i and release of final GMP as affected by heating temperature. It is clear that the rate of GMP released was affected by heating temperature. There was a progressive reduction in V_i and GMP released with increasing temperature in the range from 60 to 90 °C and then a sudden increase at 98 °C (data not shown in Figure 3A,B), the effect being more pronounced in the case of V_i than in the GMP values. The maximum reduction in V_i (about 40%) and the release of GMP (about 25%) occurred between 85 and 90 °C. The results are concordant with earlier observations (Long et al., 1963) that the degree of interaction between β-Lg and κ-C reaches a maximum at 85 °C and decreases at 99 °C. However, the increase in chymosin hydrolysis of heated casein micelles and β-Lg at 98 °C (or decrease in interaction of β-Lg with κ-C) may reflect –SH and –SS– degradation at higher temperatures (Hutton and Patton, 1952; Watanabe and Klostermeyer, 1976).

Mills (1976) reported that β-Lg undergoes heat-induced conformational changes leading to the exposure of tryptophan groups to the polar environment. Watanabe and Klostermeyer (1976) observed a decrease of –SH groups and an increase of –SS– groups with an increase in heating temperature (60–90 °C) of β-LgA at pH 6.9. Since both hydrophobic bonds and –SH and –SS– interchange reactions are involved in the heat-induced interaction of β-Lg with κ-C (Sawyer, 1969; Dziuba, 1979; Smits and van Brouwershaven, 1980; Doi et al., 1983; Haque and Kinsella, 1988), it is conceivable that the conformational changes leading to the exposure of hydrophobic groups to the polar environment during heating of β-Lg

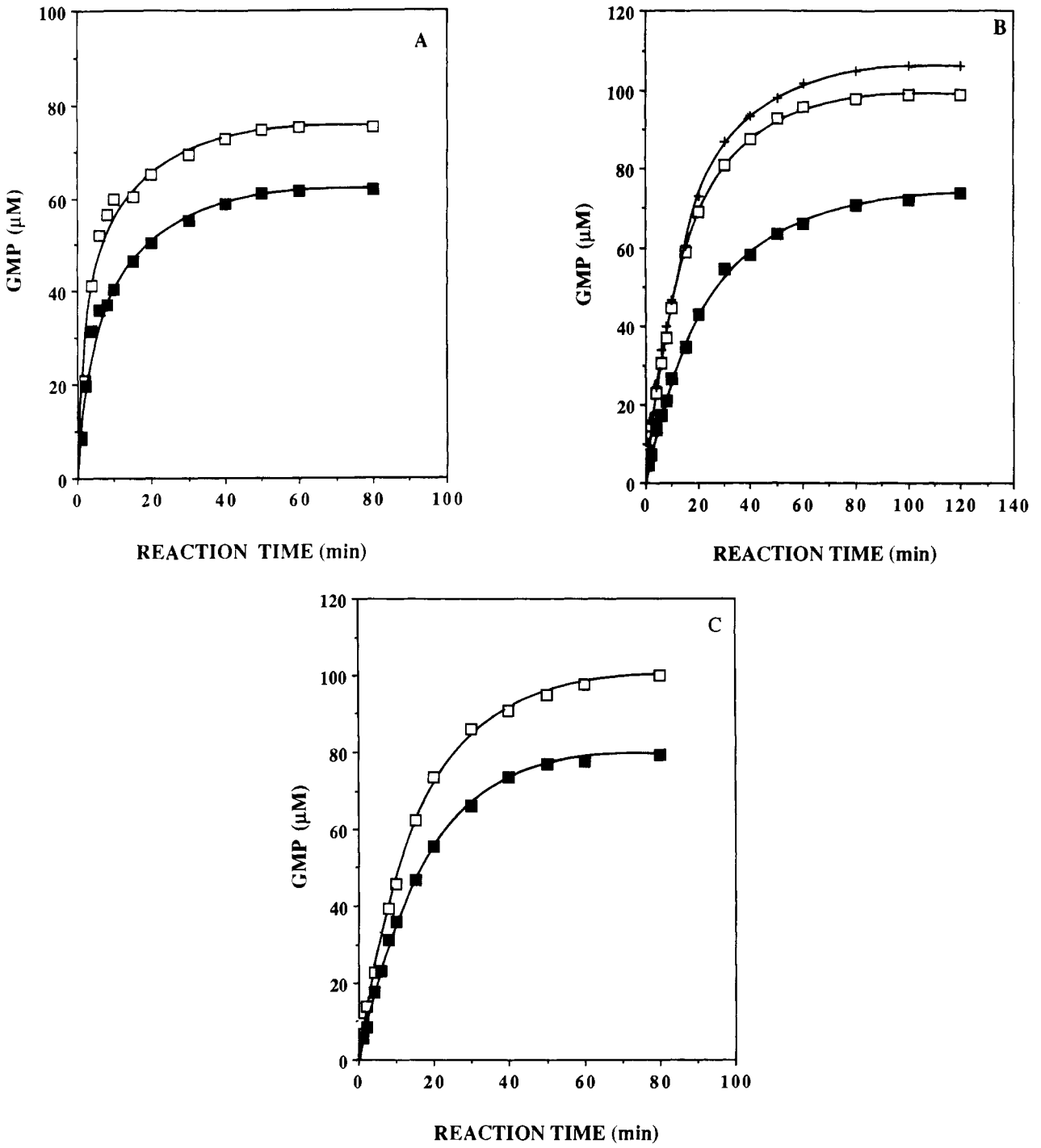

Figure 2. Interaction of β-lactoglobulin with κ-casein in heated (85 °C for 15 min, pH 6.8) solutions of κ-casein and β-lactoglobulin and of casein micelles and β-lactoglobulin and heated (85 °C for 15 min) skim milk as assessed by chymosin hydrolysis. Chymosin: 5 μg/10 mL of substrate. A: κ-casein + β-lactoglobulin, unheated (□); κ-casein + β-lactoglobulin, heated (■). B: casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated (■); casein micelles, heated (+). C: skim milk, unheated (□); skim milk, heated (■).

may favor the formation of hydrophobic interactions with apolar groups of κ-C on the micelles and subsequently to the formation of intermolecular -SS- bonds, which probably stabilize the complex. A decrease in V_i and the final GMP released at the end of 120-min hydrolysis with an increase in heating temperature from 60 to 90 °C observed in the present study is consistent with the changes in conformation (Mills, 1976) and the increase in -SS- bond content of β-Lg (Watanabe and Klostermeyer, 1976) with an increase in temperature. The results suggested that the interaction of β-Lg with κ-C on intact micelles is initiated at 60 °C and reaches a maximum at 85 °C. Interestingly, the kinetics of exposure of the buried -SH group by dissociating β-Lg dimer is rapid above 60 °C (Kella and Kinsella, 1988). Hence, a heating temperature of 85 °C was used in all the subsequent experiments.

Effect of Heating Time. The effect of heating time on chymosin kinetics was followed by heating the reaction mixture containing casein micelles and β-Lg at 85 °C for 1, 2, 6, and 10 min (Figure 4A,B). A decrease in V_i and the release of GMP occurred with an increase in heating time up to 10 min. However, a heating time of 15 min did not result in further decrease of V_i and GMP released (data not shown). A reduction as high as 38% in V_i and 28% in the GMP released was observed at 10-min heating time. Hence, a heating time of 10 min at

Table I. Initial Velocity of and Total Amount of Glycomacropeptide Released by the Chymosin Hydrolysis of Heated (85 °C for 15 min; pH 6.8) Solutions of κ-Casein and β-Lactoglobulin and of Casein Micelles and β-Lactoglobulin and Heated (85 °C for 15 min) Skim Milk

	V_i, μmol/s	GMP, μL
κ-casein + β-lactoglobulin (unheated)	15.83×10^{-2}	75.30[a]
κ-casein + β-lactoglobulin (heated)	12.67×10^{-2}	61.80[a]
casein micelles + β-lactoglobulin (unheated)	8.17×10^{-2}	99.00[b]
casein micelles (heated)	9.00×10^{-2}	106.50[b]
casein micelles + β-lactoglobulin (heated)	5.17×10^{-2}	73.80[b]
skim milk (unheated)	9.17×10^{-2}	99.90[a]
skim milk (heated)	6.50×10^{-2}	79.20[a]

[a] At the end of 80-min hydrolysis. [b] At the end of 120-min hydrolysis.

85 °C was used for all the subsequent experiments. Watanabe and Klostermeyer (1976) reported a decrease in -SH groups and an increase in the -SS- groups of β-LgA with heating at 75 and 85 °C (pH 6.9), which reached a maximum between 10 and 15 min. Therefore, heating of casein micelles and β-Lg mixture for 10 min at 85 °C to attain maximum complex formation seemed in order.

Effect of β-Lg Concentration. The effect of varying the concentration of β-Lg from 0.05 to 0.50% and heating at 85 °C for 10 min on the time course of chymosin hydrolysis of casein micelles is summarized in Figure 5A. There was a progressive decrease in rate of hydrolysis with increase in β-Lg concentration from 0.05 to 0.50%. The decrease in V_i and release of GMP is proportional to the concentration of β-Lg, with maximum decreases of 52 and 46% of V_i and GMP, respectively, at 0.5% β-Lg concentration (Figure 5B).

Long et al. (1963) reported that the degree of interaction of β-Lg with κ-C depended on the ratio of the two proteins. As the proportion of β-Lg was increased, a higher percentage of protein interacted at 85 °C to form the complex. The results obtained in the present study are in concurrence with those reported by Long et al. (1963).

Wheelock and Kirk (1974) found that the release of peptides decreased with increasing concentration of β-Lg and reached a constant value at 30 mg of β-Lg/100 mL of micelle suspension, which is far less than the native β-Lg concentration (320 mg/100 mL) in milk. β-Lg undergoes some self-aggregation before forming a complex with κ-C (Sawyer, 1969; Dziuba, 1979; Haque et al., 1987). The self-aggregation of β-Lg molecules would be concentration dependent. Thus, at high mixing ratios (i.e., high β-Lg concentrations), the aggregated form of β-Lg would be favored and the amount of β-Lg–κ-C complex would increase. In the present study, this trend is reflected in the decreased rate of chymosin hydrolysis of heated casein micelles with increasing concentration of β-Lg.

Effect of pH Adjustment before Heating. Smits and van Brouwershaven (1980) reported that when a mixture of casein micelles and β-Lg was heated at 90 °C, the amount of β-Lg bound to casein micelles was decreased at pH 7.3 and increased at pH 5.8. Creamer and Matheson (1980) concluded that denatured whey proteins do not attach to the micelles after heating at pH values >6.8, which may suggest that κ-C is then no longer involved in the -SH and -SS- interchange reactions. Hence, the effects of pH on the chymosin hydrolysis were checked by heating the casein micelles and β-Lg mixture at pHs 6.0 and 7.5, followed by adjusting the pH to the original value, i.e., 6.8 before adding chymosin.

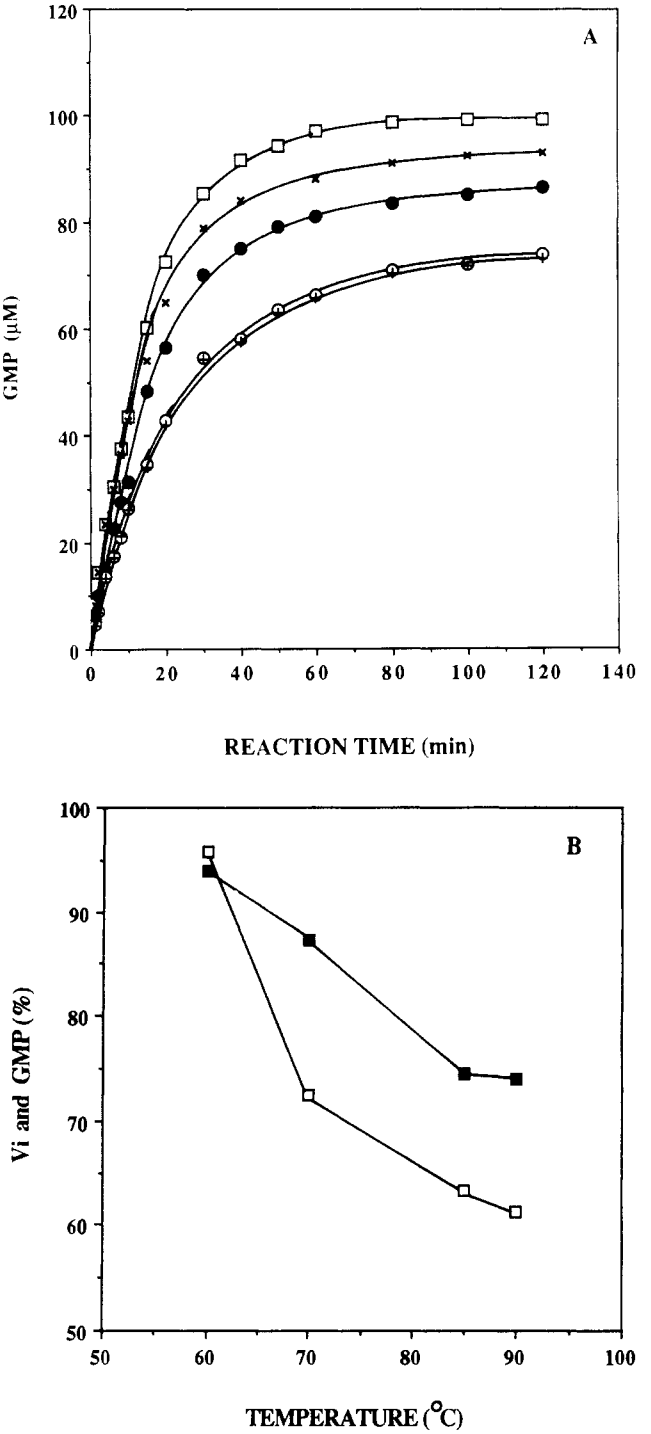

Figure 3. Effect of heating temperature on the interaction of β-lactoglobulin with κ-casein in micelles as shown by kinetics of chymosin hydrolysis of κ-casein. Conditions: heating time, 30 min; pH 6.8; chymosin, 5 μg/10 mL of substrate. A: casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated at 60 °C (×), 70 °C (●), 85 °C (○), and 90 °C (+). B: Plot of initial velocity, V_i (□), and glycomacropeptide, GMP (■), vs temperature. Values for unheated casein micelles + β-lactoglobulin were set at 100%.

The results (Figure 6A,B) indicate that V_i and the release of GMP decreased with an increase in heating pH, although the effect was more pronounced in the case of V_i than GMP. Contrary to the observations of Creamer and Matheson (1980), the decrease in chymosin hydrolysis at pH 7.5 indicates the occurrence of interaction of β-Lg with κ-C in micelles at this pH. Further, the conformational status (heat- and pH-induced) of β-Lg at pHs 6.0

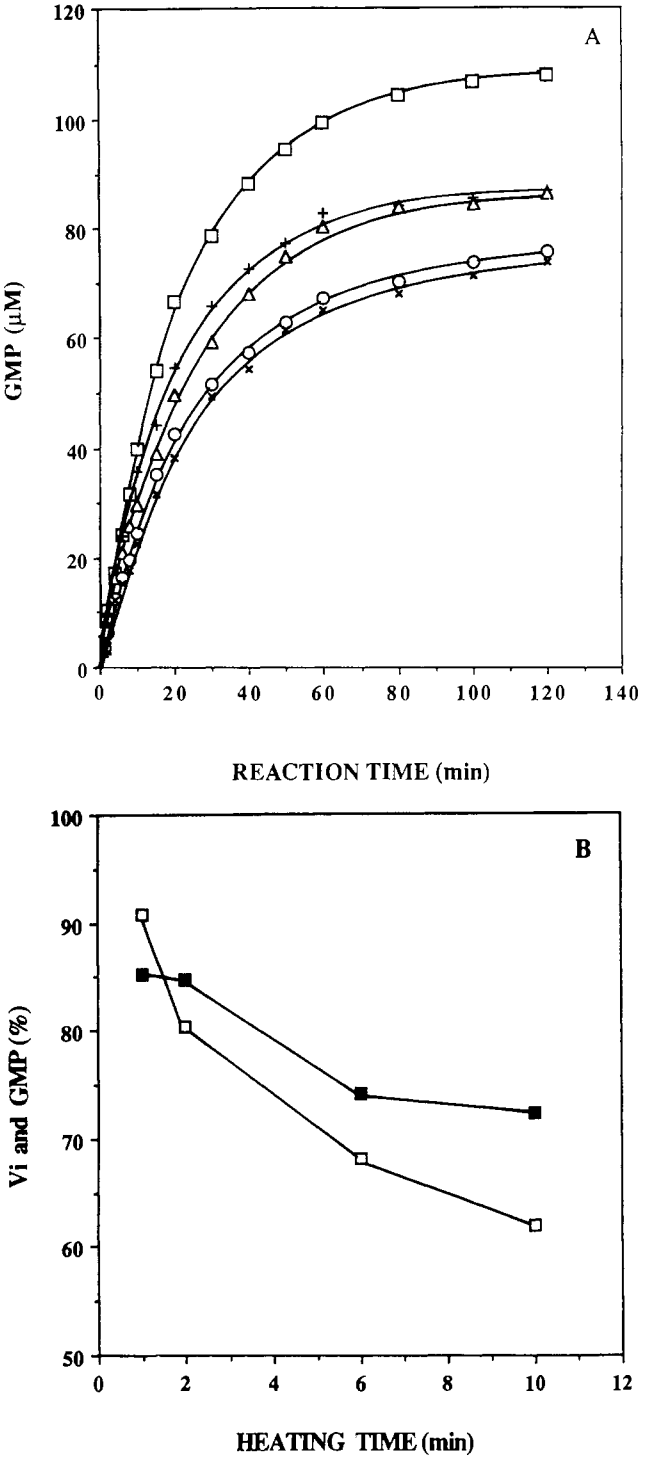

Figure 4. Effect of heating time on the interaction of β-lactoglobulin with κ-casein in micelles as shown by kinetics of chymosin hydrolysis of κ-casein. Conditions: temperature, 85 °C; pH 6.8; chymosin, 5 μg/10 mL of substrate. A: casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated for 1 min (+), 2 min (△), 6 min (○), and 10 min (×). B: Plot of initial velocity, V_i (□), and glycomacropeptide, GMP (■), vs heating time. Values for unheated casein micelles + β-lactoglobulin were set at 100%.

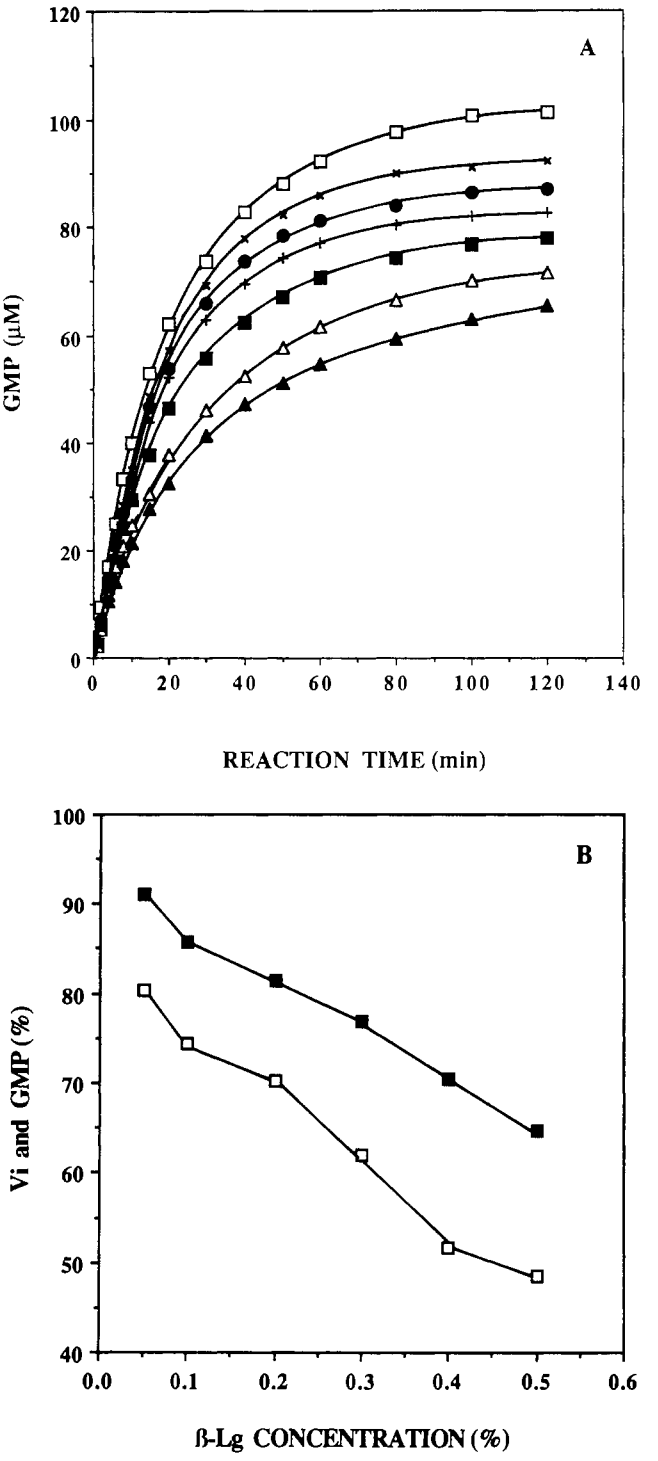

Figure 5. Effect of β-lactoglobulin concentration on interaction with κ-casein in micelles as shown by kinetics of chymosin hydrolysis of κ-casein. Conditions: heating, 85 °C for 10 min; pH 6.8; chymosin, 5 μg/10 mL of substrate. A: casein micelles + β-lactoglobulin, unheated (□); casein micelles heated with 0.05% β-lactoglobulin (×), 0.10% β-lactoglobulin (●), 0.20% β-lactoglobulin (+), 0.30% β-lactoglobulin (■), 0.40% β-lactoglobulin (△), and 0.50% β-lactoglobulin (▲). B: Plot of initial velocity, V_i (□), and glycomacropeptide, GMP (■), vs β-lactoglobulin concentration. Values for unheated casein micelles + β-lactoglobulin were set at 100%.

and 7.5 (McKenzie, 1971) also suggest that it is capable of forming hydrophobic as well as –SH and –SS– interchange reactions with κ-C when heated at 85 °C.

Effect of pH Adjustment after Heating. Roefs et al. (1985) and van Hooydonk et al. (1986b) summarized the changes occurring in casein micelles as pH is decreased from 6.8 to 4.6, with maximum changes occurring at pH 5.4. These include decrease in hydrodynamic diameter, increase in apparent voluminosity, and dissociation of β-, κ-, and α_s-caseins from micelles. These changes may affect chymosin hydrolysis of the heated mixture of casein micelles and β-Lg when the pH of the reaction mixture is lowered after heating. van Hooydonk et al. (1987) and

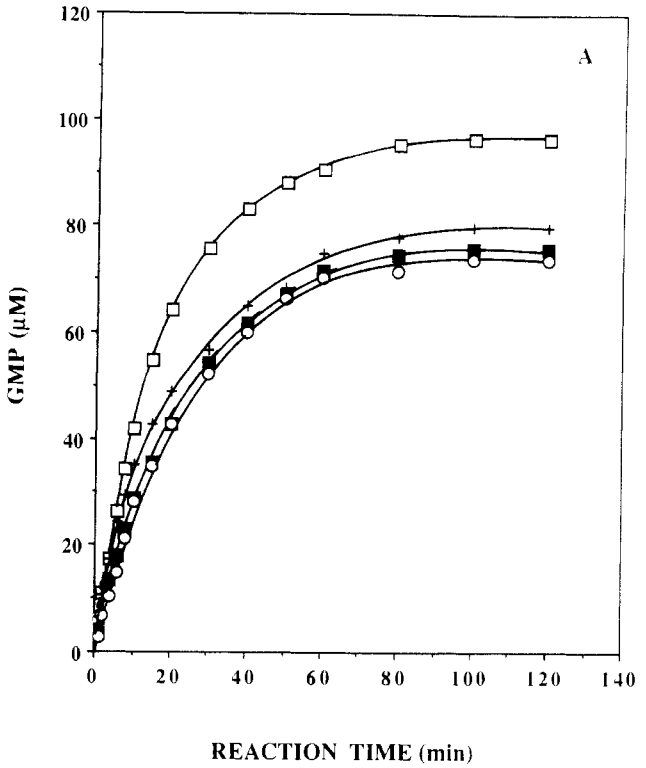

Figure 6. Effect of heating pH on the interaction of β-lactoglobulin with κ-casein in micelles as shown by kinetics of chymosin hydrolysis of κ-casein. Conditions: heating at 85 °C for 10 min; chymosin, 5 μg/10 mL of substrate. A: casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated at pH 6.0 (+), pH 6.8 (■), and pH 7.5 (○). B: Plot of initial velocity, V_i (□), and glycomacropeptide, GMP (■), vs heating pH. Values for unheated casein micelles + β-lactoglobulin were set at 100%.

Singh et al. (1988) reported that acidification and neutralization of heated milk improved the rennet clotting time. We checked this by decreasing the pH of the heated and cooled casein micelles and β-Lg mixture to 5.8 and immediately readjusting to pH 6.8 before the addition of chymosin. The results (Figure 7) show that recycling of pH improved the final GMP released by about 13%, although it had little effect on the V_i. The adjustment

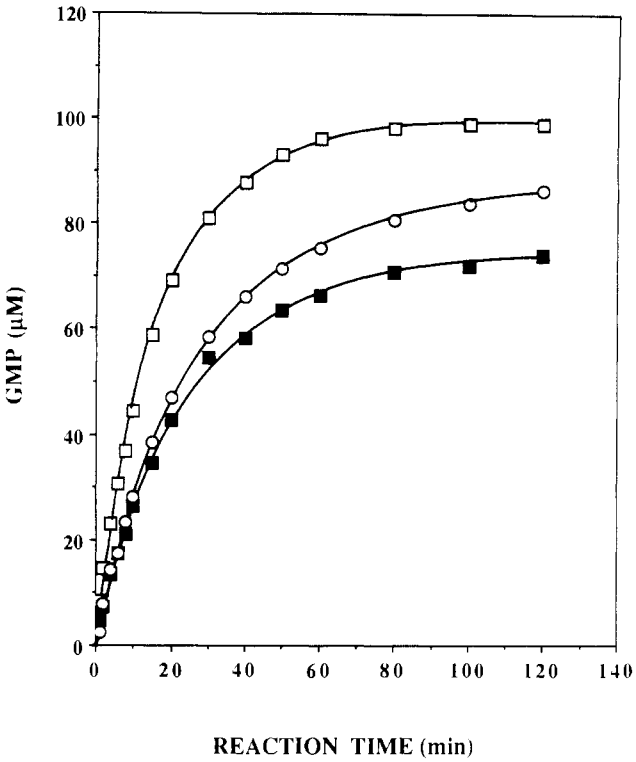

Figure 7. Effect of pH adjustment after heating a mixture of casein micelles and β-lactoglobulin (85 °C for 10 min, pH 6.8) on the kinetics of chymosin hydrolysis of κ-casein (chymosin, 5 μg/10 mL of substrate). Key: casein micelles + β-lactoglobulin, unheated (□); casein micelles + β-lactoglobulin, heated at pH 6.8 (■); casein micelles + β-lactoglobulin, heated at pH 6.8, dropped to pH 5.8, and readjusted to pH 6.8 before chymosin addition (○).

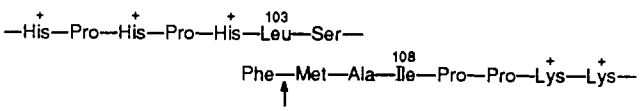

Figure 8. Amino acid sequence of κ-casein around the chymosin-sensitive Phe^{105}–Met^{106} bond.

of pH described above may, by altering the structure of micelles, cause some dissociation of the complexed β-Lg and render some of the κ-C accessible or susceptible to chymosin, thus increasing the rate of hydrolysis.

Chymosin, an acid proteinase, consists of two domains separated by a deep cleft in which the active sites for substrate binding are located. Two aspartic residues play a major role in the catalytic mechanism of the enzyme (Foltmann, 1981). The optimum pH for general proteolysis of peptides is around 4.0 (Garnier et al., 1968), whereas the optimum for the specific cleavage of the highly susceptible Phe^{105}–Met^{106} bond is near pH 5.4 for isolated κ-C and fragments thereof (Garnier et al., 1968; Visser, 1981) and around pH 6.0 in milk (van Hooydonk et al., 1986a).

The amino acid sequence of κ-C around the chymosin-sensitive Phe^{105}–Met^{106} bond is shown in Figure 8. An extended β-sheet structure is predicted around the Phe^{105}–Met^{106} bond (Raap et al., 1983). The GMP part of the molecule has an excess of negative charges, probably preventing its folding into a compact structure (Raap et al., 1983). On the basis of the results of kinetics of the reaction between chymosin and peptide substrates representing parts of κ-C sequence around the Phe^{105}–Met^{106} bond, Visser (1981) concluded that residues 98–112 of κ-C include the structural elements required to facilitate the interaction of κ-C with the active site of

chymosin near neutral pH. Payens and Both (1980) postulated that the positive cluster around Phe^{105}–Met^{106} bond interacts with counterparts near the enzyme's active site, thus making the Phe^{105}–Met^{106} bond more accessible to enzymic attack. An investigation of Visser et al. (1977) on the contribution of individual amino acid residues around the Phe^{105}–Met^{106} bond can be summarized as follows. Hydrophobic side chains of Leu^{103} and Ile^{108} form additional sites of enzyme–substrate interaction. The hydroxyl group of Ser^{104} is strongly involved, probably via hydrogen bond formation, both in binding and in the catalytic reaction. Additionally, the Pro residues at positions 101, 109, and 110, His^{102}, and Lys^{111} appear to be important for substrate activity. Thus, it may be assumed that the fragment 103–108 is brought into correct position via electrostatic bonding (His^{102} and Lys^{111}) near the active site of the enzyme, whereas Pro (101, 109, 110), Leu^{103}, Ile^{108}, and Ser^{104} stabilize the substrate conformation in the enzyme–substrate complex.

Interaction of β-Lg with κ-C involving hydrophobic as well as intermolecular –SS– bonds may result in secondary interactions such as ionic and hydrogen bond formation around the Phe^{105}–Met^{106} bond region of κ-C; an increase in net negative charge of the micelles, since β-Lg carries a net negative charge of –10 at pH 6.6 (Basch and Timasheff, 1967); and steric hindrance of β-Lg associated with κ-C. Noncovalent (hydrophobic, ionic, hydrogen bonds) interactions may involve some of the abovementioned amino acid residues responsible for substrate activity, thus preventing enzyme–substrate complex formation. An increased net negative charge of the micelles may result in an increased electrostatic repulsion between the enzyme molecule and its substrate. Direct steric hindrance of β-Lg associated with κ-C may interfere with chymosin binding. 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. Thus, an increased electrostatic repulsion between the enzyme and substrate as a result of heat-induced interaction of β-Lg with micellar κ-C may be an important factor in the inhibition of chymosin hydrolysis.

In summary, inhibition of chymosin hydrolysis was dependent on heating temperature, heating time, β-Lg concentration, and heating pH. Inhibition reached a maximum at 85 °C and a heating time of 10 min at 85 °C. Concentration of β-Lg higher than that present in milk (>0.32%) enhanced the inhibition of chymosin hydrolysis. Heating pH over a range from 6.0 to 7.5 did affect the inhibition process. Dropping the pH of heated casein micelles and β-Lg mixture to 5.8 and readjusting to pH 6.8 before chymosin addition decreased to some extent the inhibition of enzymic reaction. In the subsequent paper the effects of various agents in minimizing the interaction between β-Lg and κ-C are reported.

ACKNOWLEDGMENT

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LITERATURE CITED

Armstrong, J. M.; McKenzie, H. A.; Sawyer, W. H. On the Fractionation of β-Lactoglobulin and α-Lactalbumin. *Biochim. Biophys. Acta* **1967**, *147*, 60–72.

Basch, J. J.; Timasheff, S. Hydrogen Ion Equilibrium of the Genetic Variants of Bovine β-Lactoglobulin. *Arch. Biochem. Biophys.* **1967**, *118*, 37–47.

Beeby, R. The use of Fluorescamine at pH 6.0 to Follow the Action of Chymosin on K-Casein and to Estimate This Protein in Milk. *N.Z. J. Dairy Sci. Technol.* **1980**, *15*, 99–108.

Creamer, L. K.; Matheson, A. R. Effect of Heat Treatment on the Proteins of Pasteurized Skim Milk. *N.Z. J. Dairy Sci. Technol.* **1980**, *15*, 37–49.

Doi, H.; Ibuki, F.; Kanamori, M. Heterogeneity of Reduced Bovine κ-Casein. *J. Dairy Sci.* **1979**, *62*, 195–203.

Doi, H.; Ideno, S.; Ibuki, F.; Kanamori, M. Heat-induced Complex Formation Between κ-Casein and β-Lactoglobulin. *J. Jpn. Soc. Food Nutr.* **1981**, *34*, 565–569.

Doi, H.; Ideno, S.; Ibuki, F.; Kanamori, M. Participation of the Hydrophobic Bond in Complex Formation Between κ-Casein and β-Lactoglobulin. *Agric. Biol. Chem.* **1983**, *47*, 407–409.

Domicz, W.; Dziuba, J. Studies on Casein Proteolysis. 1. Enzymic Phase of Casein Coagulation as Influenced by Heat Treatment of Milk Proteins. *Milchwissenschaft.* **1975**, *30*, 399.

Dziuba, J. The Share of Functional Casein Groups in the Formation of a Complex with β-Lactoglobulin. *Acta Aliment. Pol.* **1979**, *5*, 97–115.

Elfagm, A. A.; Wheelock, J. V. Effect of Heat on α-Lactalbumin and β-Lactoglobulin in Bovine Milk. *J. Dairy Res.* **1977**, *44*, 367–371.

Euber, J. R.; Brunner, J. R. Interaction of κ-Casein with Immobilized β-Lactoglobulin. *J. Dairy Sci.* **1982**, *65*, 2384–2387.

Foltman, B. Mammalian Milk-Clotting Proteases: Structure, Function, Evolution and Development. *Neth. Milk Dairy J.* **1981**, *35*, 223–231.

Garnier, G.; Mocquot, B.; Ribadeau-Dumas, B.; Maubois, J. L. Coagulation du Lait Par la Presure: Aspects Scientifiques et Technologiques. *Ann. Nutr. Alim.* **1968**, *22*, B495–B552.

Haque, Z.; Kinsella, J. E. Interaction Between Heated κ-Casein and β-Lactoglobulin: Predominance of Hydrophobic Interactions in the Initial Stages of Complex Formation. *J. Dairy Res.* **1988**, *55*, 67–80.

Haque, Z.; Kristjansson, M. M.; Kinsella, J. E. Interaction Between κ-Casein and β-Lactoglobulin: Possible Mechanism. *J. Agric. Food Chem.* **1987**, *35*, 644–649.

Hindle, E. J.; Wheelock, J. V. The Release of Peptides and Glycopeptide by the Action of Heat on Cow's Milk. *J. Dairy Res.* **1970**, *37*, 397–405.

Hutton, J. T.; Patton, S. The Origin of Sulfhydryl Groups in Milk Proteins and Their Contribution to "cooked" Flavor. *J. Dairy Sci.* **1952**, *35*, 699–705.

Itzhaki, R. F.; Gill, D. M. A Micro-Biuret Method for Estimating Proteins. *Anal. Biochem.* **1964**, *9*, 401–410.

Jenness, R.; Koops, J. Preparation and Properties of a Salt Solution Which Simulates Milk Ultrafiltrate. *Neth. Milk Dairy J.* **1962**, *16*, 153–164.

Karman, A. H.; van Boekel, M. A. J. S. Evaluation of the Kjeldahl Factor for Conversion of the Nitrogen Content of Milk and Milk Products to Protein Content. *Neth. Milk Dairy J.* **1986**, *40*, 315–336.

Kella, N. K. D.; Kinsella, J. E. Structural stability of β-Lactoglobulin in the Presence of Kosmotropic Salts. A Kinetic and Thermodynamic Study. *Int. J. Peptide Protein Res.* **1989**, *32*, 396–405.

Long, J. E.; van Winkle, Q.; Gould, I. A. Heat-Induced Interaction Between Crude κ-Casein and β-Lactoglobulin. *J. Dairy Sci.* **1963**, *46*, 1329–1334.

Makino, S.; Niki, R. Interaction of Sodium Dodecyl Sulfate and of Non-ionic Detergents With S-Carboxyamidomethyl-κ-Casein. *Biochim. Biophys. Acta* **1977**, *495*, 99–109.

Marshall, R. J. Increasing Cheese Yields by High Heat Treatment of Milk. *J. Dairy Sci.* **1986**, *53*, 313–322.

Matterella, N.; Richardson, T. Adsorption of Positively-Charged β-Lactoglobulin Derivatives to Casein Micelles. *J. Dairy Sci.* **1982**, *65*, 2253–2258.

McKenzie, H. A. β-Lactoglobulins. In *Milk Proteins-Chemistry and Molecular Biology*; McKenzie, H. A., Ed.; Academic: New York, 1971; Vol. 2, p 317.

Mills, O. E. Effect of Temperature on Tryptophan Fluorescence of β-Lactoglobulin B. *Biochim. Biophys. Acta* **1976**, *434*, 324–332.

Ono, T.; Yada, R.; Yutani, K.; Nakai, S. Comparisons of Conformations of κ-Casein, Para-κ-Casein and Glycomacropeptide. *Biochim. Biophys. Acta* **1987**, *911*, 318–325.

Parnell-Clunies, E.; Kakuda, Y.; Irwine, D.; Mullen, K. Heat-Induced Changes in Milk Processed by Vat and Continuous Heating Systems. *J. Dairy Sci.* **1988**, *72*, 1472–1483.

Payens, T. A. J.; Both, P. Enzymic Clotting Process. IV. The Chymosin-Triggered Clotting of Para-κ-Casein. *In Bioelectrochemistry: ions, surfaces, membranes*; Blank, E., Ed.; Advances in Chemistry Series 188; American Chemical Society: Washington, 1980; pp 130–141.

Raap, J.; Kerling, K. E. T.; Vreeman, H. J.; Visser, S. Peptide Substrates for Chymosin (Rennin): Conformational Studies of κ-Casein and Some κ-Casein-Related Oligopeptides by Circular Dichroism and Secondary Structure Prediction. *Arch. Biochem. Biophys.* **1983**, *221*, 117–124.

Roefs, S. P. F. M.; Walstra, P.; Dalgleish, D. G.; Horne, D. S. Preliminary Note on the Change in Casein Micelles Caused by Acidification. *Neth. Milk Dairy J.* **1985**, *39*, 119–122.

Sawyer, W. H. Complex Between β-Lactoglobulin and κ-Casein. A review. *J. Dairy Sci.* **1969**, *52*, 1347–1355.

Shalabi, S. I.; Wheelock, J. V. Effect of Sulfhydryl-Blocking Agents on the Primary Phase of Chymosin Action on Heated Casein Micelles and Heated Milk. *J. Dairy Sci.* **1977**, *44*, 351–355.

Singh, H.; Shalabi, S. I.; Fox, P. F.; Flynn, A.; Barry, A. Rennet Coagulation of Heated Milk: Influence of pH Adjustment Before or After Heating. *J. Dairy Sci.* **1988**, *55*, 205–215.

Smits, P.; van Brouwershaven, J. H. Heat-Induced Association of β-Lactoglobulin and Casein Micelles. *J. Dairy Sci.* **1980**, *47*, 313–325.

Snoeren, T. H. M.; van der Spek, C. A. The Isolation of a Heat Induced Complex from UHTST Milk. *Neth. Milk Dairy J.* **1977**, *31*, 352–355.

Townend, R.; Herkovits, T. T.; Swaisgood, E. H.; Timasheff, S. N. The Solution Properties of β-Lactoglobulin C. *J. Biol. Chem.* **1964**, *239*, 4196–4201.

van Hooydonk, A. C. M.; Oleiman, C. A Rapid and Sensitive High-Performance Liquid Chromatography Method of Following the Action of Chymosin in Milk. *Neth. Milk Dairy J.* **1982**, *36*, 153–158.

van Hooydonk, A. C. M.; Boerrigter, I. J.; Hagedoorn, H. G. pH-Induced Physico-Chemical Changes of Casein Micelles in Milk and Their Effect on Renneting. 2. Effect of pH on Renneting of Milk. *Neth. Milk Dairy J.* **1986a**, *40*, 297–313.

van Hooydonk, A. C. M.; Hagedoorn, H. G.; Boerrigter, I. J. pH-Induced Physico-Chemical Changes of Casein Micelles in Milk and Their Effect on Renneting. 1. Effect of Acidification on Physico-Chemical Properties. *Neth. Milk Dairy J.* **1986b**, *40*, 281–296.

van Hooydonk, A. C. M.; De Koster, P. F.; Boerrigter, I. J. The Renneting Properties of Heated Milk. *Neth. Milk Dairy J.* **1987**, *41*, 3–18.

Visser, S. Proteolytic Enzymes and Their Action on Milk Proteins. A Review. *Neth. Milk Dairy J.* **1981**, *35*, 65–88.

Visser, S.; van Rooijen, P. J.; Schattenkerk, C.; Kerling, K. E. T. Peptide Substrates for Chymosin (Rennin). Kinetic studies with Bovine κ-Casein-(103-108)-Hexapeptide. *Biochem. Biophys. Acta* **1977**, *481*, 171–176.

Watanabe, K.; Klostermeyer, H. Heat-Induced Changes in Sulfhydryl and Disulfide Levels of β-Lactoglobulin A and the Formation of Polymers. *J. Dairy Res.* **1976**, *43*, 411–418.

Wheelock, J. V.; Kirk, A. The Role of β-Lactoglobulin in the Primary Phase of Rennin Action on Heated Casein Micelles and Heated Milk. *J. Dairy Res.* **1974**, *41*, 367–372.

Wilson, G. A.; Wheelock, J. V. Factors Affecting the Action of Rennin in Heated Milk. *J. Dairy Res.* **1972**, *39*, 413–419.

Wilson, G. A.; Wheelock, J. V.; Kirk, A. The Effect of Reduction and Alkylation on the Primary Phase of Rennin Action on Unheated and Heated Milk. *J. Dairy Res.* **1974**, *41*, 37–44.

Zittle, C. A.; Custer, J. H. Purification and Some of the Properties of α_s-Casein and κ-Casein. *J. Dairy Sci.* **1963**, *46*, 1183–1188.

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Substitution of Pyrazines by Aldehydes in Model Systems

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The effect of long-chain aldehydes on the formation of long-chain alkyl-substituted pyrazines was investigated in model systems of acetol and ammonium acetate with and without the addition of pentanal or hexanal. When the systems were reacted at 100 °C for 4 h, 2,5-dimethyl-3-pentylpyrazine and 2,6-dimethyl-3-pentylpyrazine were formed in the model system with added pentanal, and the corresponding hexylpyrazines were formed in the hexanal system. Other pyrazines, including 2,3,5-trimethylpyrazine, 2-methyl-5(or 6)-ethylpyrazine, 2,5-dimethyl-3-ethylpyrazine, 2,6-dimethyl-3-ethylpyrazine, 2,5-dimethyl-3-allylpyrazine, 2,5-dimethyl-3-propenylpyrazine, 2,3,5-trimethyl-6- butylpyrazine, 2,3,5-trimethyl-3-pentylpyrazine, 2,3,5-trimethyl-3-hexylpyrazine, 2,5-dimethyl-3-propylpyrazine, and 2,6-dimethyl-3-propylpyrazine, were also obtained from the model systems. Formation pathways are proposed for some of these pyrazines.

The occurrence and formation of pyrazines in foods have been studied extensively in the last 25 years. A comprehensive review of these studies has been prepared by Maga (1982). Many of these studies have focused on the investigation of pyrazine precursors, especially those carbohydrate and amino acid sources most active in the Maillard reaction.

Interest in the influence of lipids on pyrazine formation has recently been generated by the identification of long-chain alkyl-substituted heterocyclic compounds in foods and in model systems. Various nitrogen- and sulfur-containing heterocyclic products with long-chain alkyl substituents have been detected in model systems (Boelens et al., 1974; Henderson and Nawar, 1981), in fried chicken extracts (Tang et al., 1983; Hartman et al., 1984), and in french-fried potato extracts (Carlin et al., 1986;

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