ARTICLES

Influence of pH on Chymosin Action in Solutions of Different κ-Casein Variants

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Chymosin hydrolysis of κ-casein variants A, B, or AB or heated (85 °C × 10 min) mixtures of κ-casein variants plus β-lactoglobulin A, B, or AB in buffers at pH 6.7, 6.4, 6.0, 5.6, or 5.3 were monitored spectrophotometrically at 420 nm by reaction with 2,4,6-trinitrobenzenesulfonic acid. The genetic type of κ-casein and pH of the medium influenced the rate and extent of hydrolysis. At pH 5.3 and 5.6 κ-casein B was most extensively hydrolyzed, but at pH 6.0–6.7 the hydrolyses of the three variants were identical. κ-Casein A was most susceptible to hydrolysis (at pH 5.3) in heated solutions containing different genetic combinations of κ-casein plus β-lactoglobulin.

Keywords: *Genetic variants; proteolysis; κ-casein; β-lactoglobulin*

INTRODUCTION

The existence of different genetic types of individual proteins in milk (Eigel et al., 1984) may influence some of the technological properties of milk (Jakob and Puhan, 1992). Hydrolysis of κ-casein (κ-CN) by rennet and the subsequent coagulation of milk are perhaps the most important steps in the manufacture of rennet-coagulated cheeses. Schaar et al. (1985) reported that, at constant firmness, the cutting time for curd from κ-CN BB milk was approximately 67% of that from κ-CN AA milk. However, Marziali and Ng-Kwai-Hang (1986) found that κ-CN AA, AB, or BB milks had similar coagulation properties (i.e., rennet-clotting time, rate of firming, or curd firmness). They also suggested that milks containing different variants of β-lactoglobulin (β-LG) may exhibit different coagulation properties.

Whey proteins (e.g., β-LG) contribute little to milk coagulation unless the milk is given a high-heat treatment (Damicz and Dziuba, 1975). Severe heat treatment of milk denatures β-LG, which interacts with κ-CN (Sawyer, 1969; Haque and Kinsella, 1988) and reduces the sensitivity of κ-CN to hydrolysis by chymosin (Wheelock and Kirk, 1974; van Hooydonk et al., 1987). The denaturation of different genetic types of β-LG occurs at different temperatures (Imafidon, 1990). Also, the presence of different genetic types of κ-CN influences the denaturation of β-LG (Imafidon et al., 1991a,b), suggesting that chymosin hydrolyses of κ-CN in solutions containing different genetic types of β-LG and κ-CN may differ. Therefore, our objectives were to determine the rates and extent of hydrolysis of different genetic types of κ-CN alone or in heated solutions containing different genetic types of β-LG.

MATERIALS AND METHODS

Milk Proteins. Different genetic types (A, B, or AB) of κ-CN were prepared from acid-precipitated bovine casein according to the method of Zittle and Custer (1963) and purified as described by Ng-Kwai-Hang and Pélissier (1989). β-Lactoglobulin types (A, B, or AB) were also prepared from whey from individual cows' milk of known genetic types according to the method of Imafidon and Ng-Kwai-Hang (1992). The purities of the individual proteins were ascertained by urea–PAGE (Ng-Kwai-Hang et al., 1984).

Enzyme. Fermentation-produced chymosin, Chymax, was obtained from Pfizer Inc., Milwaukee, WI. Its concentration was determined spectrophotometrically at 277.5 nm using the extinction coefficient ($E_{277.5}^{1\%,1cm}$) of 1.53 (Raymond et al., 1973). All other reagents were obtained from Fisher Scientific (San Francisco, CA) and were of analytical grades.

Proteolysis of κ-Casein Variants Using Chymosin. A slightly modified method of Fields (1972), as described by Addeo et al. (1984), was used to follow the kinetics of hydrolysis of κ-CN variants. Whole κ-CN (A, B, or AB) (0.2%) was dissolved in buffer I [containing 50 mM trisodium citrate, 75 mM NaCl, and 0.02% (w/v) NaN_3] at pH 5.3, 5.6, 6.0, 6.4, or 6.7. The resultant solutions were filtered through a 0.2-μm membrane filter (Gelman Sciences Inc, Ann Arbor, MI), and the true concentrations of κ-CN were determined spectrophotometrically using $E_{280}^{1\%,1cm}$ of 0.95 (Swaisgood, 1982). Then, the concentration of κ-CN in each solution was adjusted to 0.5 mg/mL by diluting with buffer I at the appropriate pH. Solutions of β-LG (0.5 mg/mL) were prepared separately and their concentrations determined spectrophotometrically using $E_{280}^{1\%,1cm}$ of 0.94 (Swaisgood, 1982).

The β-LG solutions were held separately at room temperature and added to the κ-CN solution immediately before use to prevent cold interaction (Haque and Kinsella, 1988). The resultant solution (containing κ-CN and β-LG) was heated to 85 °C in a water bath, held there for 10 min, and then cooled to 30 °C in an ice bath. To 12-mL portions of κ-CN or κ-CN+β-LG solutions was added 1.5 μL of chymosin (corresponding to 1.43 μg of chymosin), and the mixture was allowed to react at 30 °C for 12 min. Hydrolysis time was limited to 12 min because the clotting time for different genetic variants of isolated κ-CN ranges from 4.06 to 7.41 min (El-Negoumy, 1972) and the coagulation time for normal milk is between 3.91 and 9.75 min, depending on the phenotypes of β-CN, κ-CN, and β-LG present (Marziali and Ng-Kwai-Hang, 1986).

At 1-min intervals, 200 μL of the reaction mixture was taken and added to 500 μL of buffer II (containing 0.1 M sodium tetraborate and 0.2 M NaOH) at pH 11.2 to stop enzymic activity. Then, 300 μL of 0.17 M 2,4,6-trinitrobenzenesulfonic

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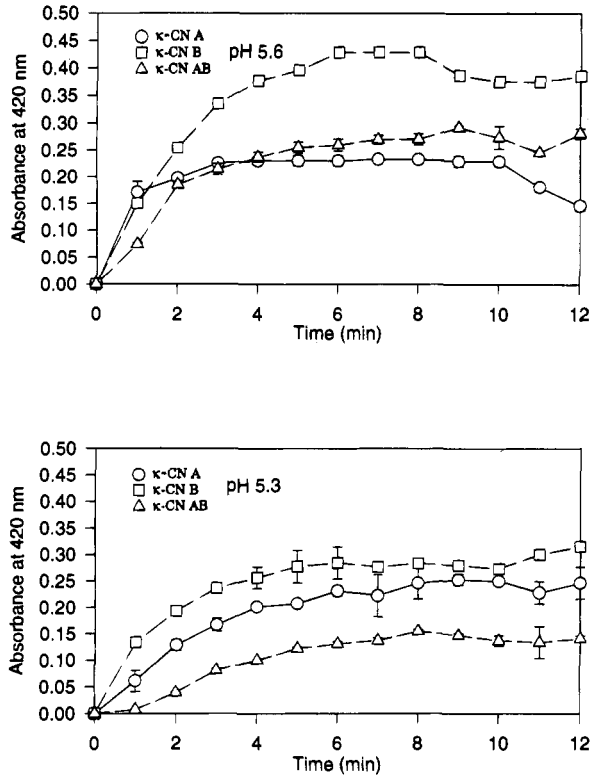

Figure 1. Mean (n = 3) A_{420nm} for TNBS-reactive groups produced from the hydrolysis (at 30 °C) of different genetic variants of κ-casein (0.55 mg/mL) by chymosin (1.43 μg) in trisodium citrate buffer at pH 5.3 or 5.6.

acid 3-hydrate (TNBS) was added while mixing. After a reaction time of 10 min at room temperature, 2 mL of freshly prepared buffer III (containing 98.5 mM NaH_2PO_4 and 1.5 mM Na_2SO_3) was added to stop the reaction and A_{420nm} determined. The experiment was replicated on three occasions using the same lots of protein variants.

Statistical Analysis. Effects of genetic polymorphism of β-LG and κ-CN on hydrolysis of κ-CN before and after high-heat treatment were examined. The data obtained were analyzed by least-squares ANOVA using the Statistical Analysis Systems package (SAS Institute Inc., Cary, NC). The model fitted was $Y_{ijmn} = \mu + \kappa\text{-CN}_i + \beta\text{-LG}_j + \text{pH}_m + T_n + e_{ijmno}$, where, Y_{ijmn} is the nth observation of A_{420nm} at different time intervals, μ is the overall mean, $\kappa\text{-CN}_i$ is the main effect of variant i of κ-CN (i = A, AB, or B), $\beta\text{-LG}_j$ is the main effect of the variant j of β-LG (j = A, AB, or B), pH_m is the main effect of pH m (m = 5.3, 5.6, 6.0, 6.4, 6.7), T_n is the main effect of time in (n = 1, 2, ..., 12 min), and e_{ijmno} is a random error term with zero mean and variance σ_e. The interaction terms were also fitted into the model: two-way interaction terms of β-LG types × κ-CN types and κ-CN types × pH were included in the appropriate sections of the models. Means and standard errors were estimated in all models. The coefficients of variation between replicates were <10.9%, and the standard errors varied from 0.000 to 0.011 for the A_{420nm} values at each time interval.

RESULTS AND DISCUSSION

Effect of pH on Hydrolysis of κ-Casein. Figures 1 and 2 depict the rate and extent of formation of TNBS-reactive products from different genetic variants of κ-CN at pH 5.3–6.7 after hydrolysis by chymosin. The A_{420nm} values obtained for the reaction at pH 5.3 or 5.6 show distinct differences (P <0.05) in the degree of hydrolysis of the different variants of κ-CN; κ-CN B was hydrolyzed most extensively (Figure 1). At pH 6.0, 6.4, or 6.7, however, the rates or extents of hydrolysis of the three genetic types of κ-CN were not statistically different,

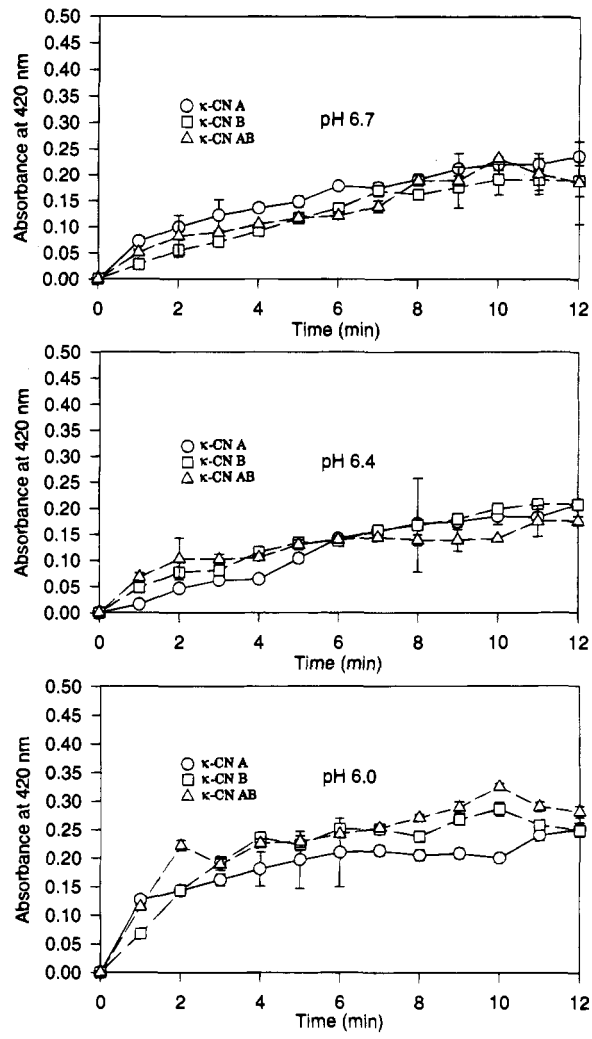

Figure 2. Mean (n = 3) A_{420nm} for TNBS-reactive groups produced from the hydrolysis (at 30 °C) of different genetic variants of κ-casein (0.55 mg/mL) by chymosin (1.43 μg) in trisodium citrate buffer at pH 6.0, 6.4, or 6.7.

although El-Negoumy (1972) reported that, in solution at pH 6.6–6.7, κ-CN B had the fastest rennet coagulation time (6.29 min), compared to 7.01 or 6.71 min, respectively, for κ-CN A or AB. Hydrolysis of the highly susceptible Phe_{105}–Met_{106} bond in isolated κ-CN (van Hooydonk et al., 1986) or its fragments (Haque and Kinsella, 1988; Visser et al., 1980) by chymosin is optimal in the pH range 5.1–5.4. However, in bulk milk, hydrolysis of κ-CN by chymosin is optimal at pH 6.0 (van Hooydonk et al., 1986). In this study, hydrolysis of the different genetic types of κ-CN was greatest at pH 5.3–5.6 (Figures 1 and 2). At pH values between 6.0 and 6.7, the differences in the degree of hydrolysis of the different κ-CN variants were not statistically significant (P > 0.05). Also, the concentrations of TNBS-reactive products released at pH 6.0–6.7 were lower than those released at pH 5.3–5.6.

pH and composition of the reaction medium may affect the structure and conformation of κ-CN which may, in turn, influence its susceptibility to chymosin. The main difference between κ-CN A and κ-CN B is the substitution of Ile and Ala at positions 136 and 148, respectively, in the former for Thr and Asp in the latter (Eigel et al., 1984; Mercier et al., 1973). The extra negative charge in the macropeptide region of κ-CN A, due to the substitution of Asp_{148} for Ala_{148} (in κ-CN B), probably increases the electrostatic or steric repulsion between the substrate and enzyme (van Hooydonk et

al., 1984) at the low pH (between 5.6 and 5.3). Consequently, the conformation of κ-CN A may be altered at low pH to expose the extra negative charge, which may cause a reduction in its susceptibility to hydrolysis by chymosin.

Effect of β-Lactoglobulin on Hydrolysis of κ-Casein. This portion of the study was carried out at pH 5.3 because preliminary experiments to determine pH effects on the rate of hydrolysis of heated (85 °C × 10 min) solutions of β-LG+κ-CN by chymosin indicated that the reaction time for which meaningful changes in A_{420nm} could be measured was >20 min. Indeed, at pH 6.7 and 10-fold increase in chymosin concentration, there was little or no change in A_{420nm} after 20 min, indicating little or no release of TNBS-reactive products. This suggests that κ-CN was resistant to hydrolysis by chymosin at high pH due to inaccessibility of the chymosin-sensitive bond in heated mixtures of κ-CN+β-LG. Damicz and Dziuba (1975) reported that β-LG was twice as susceptible to heat denaturation at pH 6.6 than at pH 5.5. The inability of chymosin to react with κ-CN in the heated solutions of κ-CN+β-LG may be due to the formation of a large heat-induced complex between the proteins as pH increased (Creamer et al., 1978).

The susceptibilities of κ-CN B or AB to hydrolysis by chymosin decreased ($P < 0.01$) in the presence of different (A, AB, or B) genetic types of β-LG as indicated by the A_{420nm} of the TNBS-reactive products formed-(Figure 3). Least-squares ANOVA showed that hydrolysis of κ-CN in heated solutions of κ-CN+β-LG at pH 5.3 was influenced significantly ($P < 0.001$) by the genetic type of each protein present. The A_{420nm} values of the TNBS-reactive groups obtained were generally lower for reaction mixtures containing heated κ-CN AB or B plus the various β-LG variants than those for κ-CN alone. Also, hydrolysis of κ-CN in heated solutions of κ-CN AB or B plus each β-LG variant (Figures 3B,C) was significantly ($P < 0.05$) lower than that in heated solutions of κ-CN A plus the different β-LG variants (Figure 3A). The average maximum A_{420nm} for hydrolysis of κ-CN A was 0.25, compared to 0.20 for the hydrolysis of κ-CN B or AB. The differences in magnitude between A_{420nm} of TNBS-reactive products from hydrolysis of κ-CN A alone and κ-CN A+β-LG A solutions were generally smaller than those from κ-CN A and κ-CN A+β-LG B (Figure 3A). This suggests that the presence of heat-denatured β-LG A in the solution inhibited κ-CN hydrolysis to a lesser extent than β-LG B. The least differences in magnitude of A_{420nm} for the TNBS-reactive products were obtained for κ-CN A and κ-CN A+β-LG AB (Figure 3A). Recently, Imafidon et al. (1991a,b) reported that polymorphic combinations of κ-CN A+β-LG B or κ-CN A+β-LG AB were most resistant to heat denaturation. Since denatured β-LG interacts with κ-CN and renders the chymosin-sensitive bond(s) on κ-CN inaccessible, the most heat resistant polymorphic combinations of κ-CN+β-LG are most likely to be hydrolyzed to the greatest extent.

Conclusions. The rate and extent of hydrolysis of κ-CN by chymosin were dependent on pH and the genetic type of κ-CN. Hydrolysis of κ-CN in heated solutions of κ-CN+β-LG was dependent on the genetic combinations of the individual proteins present. In solution alone, κ-CN B was hydrolyzed most rapidly and to the greatest extent. However, in heated solutions containing different genetic types of κ-CN+β-LG, κ-CN A was most susceptible to chymosin action at pH 5.3, suggesting that the presence of different genetic variants of β-LG and κ-CN in milk may partially explain the differences in their rates of coagulation.

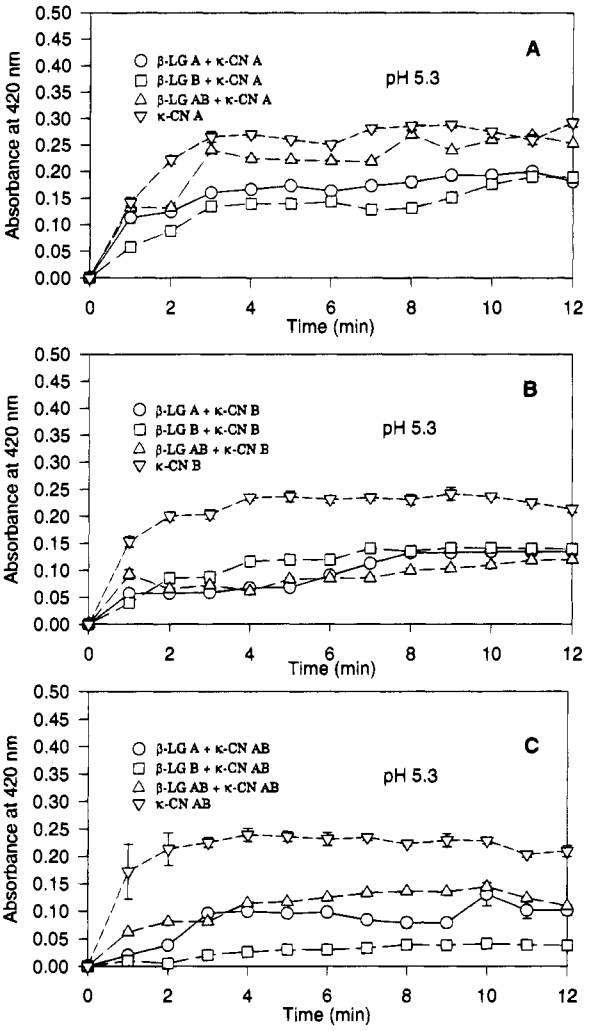

Figure 3. Mean ($n = 3$) A_{420nm} for TNBS-reactive groups produced from the hydrolysis (at 30 °C) of κ-casein in heated (85 °C × 10 min) solutions containing various genetic combinations of β-lactoglobulin (0.55 mg/mL) plus κ-casein (0.55 mg/mL) by chymosin (1.43 μg) in trisodium citrate buffer at pH 5.3: (A) solutions of κ-CN A plus β-LG A, B, or AB; (B) solutions of κ-CN B plus β-LG A, B, or AB; (C) solutions of κ-CN AB plus β-LG A, B, or AB.

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