Rennet Coagulation of Casein Micelles and Heated Casein Micelles: Importance of Steric Stabilization after κ-Casein Proteolysis

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The kinetics of aggregation of *para*-casein micelles (*p*CM) and of heated *para*-casein micelles (H*p*CM) were studied and compared by estimating the initial rate of aggregation from the initial rate of turbidity increase. Aggregation of both *p*CM and H*p*CM suspensions appeared as cases of slow Brownian flocculation, with a steric contribution to the suspension's stability which could explain their aggregation behavior, including the presence of the Berridge effect at low temperatures. Glycerol addition to the medium, besides the reduction in aggregation rate by increase of medium viscosity, produced an initial aggregation rate increase, probably by interaction with the proteins of the micelle external layer. The differences observed between *p*CM and H*p*CM aggregation rate could be related to the changes introduced by high heating in the surface structure of the micelles.

Keywords: *Heated casein micelles; steric stabilization; rennet coagulation*

INTRODUCTION

Rennet milk coagulation starts with a proteolytic phase, catalyzed by rennet, with the cleavage of the surface κ-casein of the casein micelles (CM) to *para*-κ-casein and soluble caseinomacropeptides (CMP). The resultant *para*-casein micelles (*p*CM) spontaneously aggregate in a second phase of the process (Walstra, 1990).

Heating of milk at high temperatures has been shown to improve the yield of dry solids in cheese obtained by rennet milk coagulation, by further incorporation of denatured whey proteins (Dzurec and Zall, 1985; Leaver et al., 1995). However, heating of milk results also in both longer rennet coagulation time (RCT) and weaker gels (Van Hooydonk et al., 1987).

At temperatures above 70 °C most of the whey proteins undergo progressive denaturation. Some of them, especially β-lactoglobulin (β-lg), interact with the micellar κ-casein via the formation of intermolecular disulfide bridges and perhaps other less specific forms of interactions, such as hydrogen bonding and electrostatic interactions among opposite charges (Dzurec and Zall, 1985; Pappas, 1992). The increase in RCT has been related to the extent of β-lg denaturation and hence to β-lg−κ-casein interaction (Van Hooydonk, 1987; Dalgleish, 1990).

Several authors have reported that heating of milk affects the primary phase of coagulation, inhibiting both the initial velocity of the hydrolysis and the maximum amount of CMP released (Leaver et al., 1995; Reddy and Kinsella, 1990). This behavior has also been related to the formation of the β-lg−κ-casein complex, in which the rennet-sensitive bond (Phe−Met) becomes less accessible to the enzyme than in κ-casein.

Furthermore, heating at relatively high temperatures produces modifications both in size and in surface characteristics of CM. CM average diameter is increased with reduction of the range of CM size, while CM surface becomes ragged and forms many appendages, as observed by electron microscopy (Mohammad and Fox, 1987). Since the stability of *p*CM in suspension could involve a steric component (Walstra, 1990; Gatti et al., 1995), an alteration of CM surface could be associated with stability modifications and, hence, with changes in the kinetics of the second stage of rennet coagulation.

In the present work we have used enzyme/substrate concentration ratios high enough to complete the primary phase of casein micelle rennet coagulation in the initial times (within the first 30 s), separating it from the second phase, to be able to study the kinetics of this last approach already used to study *p*CM aggregation (Carlson et al., 1987; Gatti et al., 1996; Gatti and Pires, 1995). Turbidity measurements were used to study and compare the aggregation processes of *p*CM and heated *p*CM (H*p*CM). The kinetic order of the processes was studied by working at different CM initial concentrations. Since *p*CM aggregation is usually considered as a case of Brownian aggregation, the effect of temperature and viscosity of the medium on the kinetics was also studied, modifying the medium viscosity by glycerol addition.

MATERIALS AND METHODS

Preparation of Milk Samples. Milk suspensions were prepared from bovine commercial, low-heat, nonfat dried milk (MOLICO, Société des Produits Nestlé S.A., Vevey, Switzerland) reconstituted to 10% (w/v) concentration in 5 mM $CaCl_2$, and their pH values were adjusted to 6.4 by addition of 1.5 M HCl. Protein concentration was determined according to Kuaye's method (Kuaye, 1994). Heated casein micelles were obtained from milk reconstituted, heated 10 min at 95 °C, and filtered through glass fiber filters (Whatman GF/A) (Horne, 1986), with previous skimming of the floating precipitate of denatured whey proteins to avoid filter obstruction. Heated and nonheated milks, conserved overnight at 4 °C, were equilibrated at the temperature at which aggregation was going to be studied before their dilution. Dilutions of heated (HCM) and nonheated milk (CM) were prepared in 10 mM imidazole and 5 mM $CaCl_2$ at pH 6.4 at casein concentrations in the range 0.08−0.44 g/L. The dilutions were allowed to equilibrate for 2 h at the working temperature to reach constant turbidity values. In the studies of the effect of CM concentration the temperature for coagulation was 35 °C. The effect of temperature on coagulation kinetics was studied in

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the 17−45 °C temperature range. The effect of medium viscosity was studied in the (0.77−2.42) × 10^{-2} g cm^{-1} s^{-1} viscosity range at 24, 27, 31, and 35 °C. The different viscosity values were obtained by adding glycerol at different concentrations to the coagulation buffer. The viscosity was measured using a Cannong-Fenske viscosimeter. Commercial liquid rennet with a strength of 100 RU was a gift of COTAR S.A. (Argentina) (Gatti et al., 1995). One hundred microliters of a 1/8 (v/v) dilution of rennet was added to 3 mL of milk dilution of about 0.3 g/L concentration. For samples with casein concentration different from 0.3 g/L, the rennet dilution was added in amounts adequate to maintain the same enzyme/substrate concentration ratio.

Aggregation Kinetics. According to several authors (Dalgleish, 1979; Payens, 1979; Van Hooydonk and Walstra, 1987; Carlson, 1987; Green and Morant, 1981), a minimal value of κ-casein conversion to *para*-κ-casein has to be reached in the proteolytic first step of enzymic CM coagulation for *p*CM aggregation to occur. This minimal value oscillates, according to different authors, in the range of 60−85% of the total conversion. After this value is reached, aggregation overlaps the residual κ-casein proteolysis until the end of this last stage. Different kinetic models have been proposed for the complete process, including usually the analysis of the first step by a first-order Michaelis−Menten kinetics, and the Smoluchowski's approach for Brownian aggregation kinetics at initial times, for the *p*CM aggregation step (Payens, 1979; Van Hooydonk and Walstra, 1987; Darling and Van Hooydonk, 1981). However, the interpretation by such complete models of experimental measures made by simple techniques such as turbidimetry or viscosimetry would require the knowledge of parameters not always easy to know in different conditions.

If the complete process is followed by measuring turbidity as a function of time, a turbidity decrease will be observed during the proteolysis step because of particle size reduction, while a turbidity increase will be associated with the aggregation process. Both effects will be simultaneously present when the end of the first step overlaps the beginning of the second one, making the observation of the initial times of aggregation very difficult or impossible.

A simpler approach proposed by several authors (Dalgleish, 1983; Carlson et al., 1987; Gatti et al., 1996) consists in the separation of the proteolytic step from the aggregation one by working in adequate conditions. If the primary stage is completed before the start of the aggregation, this second stage can be studied directly at its initial times.

The approach proposed by Carlson (1987), and applied in this work, consists in the use of an enzyme excess to complete the first step practically before the start of the aggregation, which appears then as the rate-limiting step. The *p*CM aggregation kinetics can be studied in these conditions by the initial rate of doublet formation, as proposed by Smoluchowski.

The disappearance of primary particles from a dilute homogeneous suspension to form doublets will initially follow a second-order kinetics. At initial times when $N(t)$, the number density of primary particles, is close to its initial value, N_0, the rate of disappearance of primary particles will be

$$[\partial N(t)/\partial t]_0 = k_2 {N_0}^2 \tag{1}$$

where k_2 is a second-order rate constant. In conditions in which the first proteolytic step of milk coagulation has been completed at the beginning of the process, practically before the start of aggregation, the initial time of aggregation can be defined as the time at which 100% of the caseinomacropeptides have been released.

Working at constant N_0, the aggregation rate constant can be estimated by the initial rate of increase of the relative turbidity $(1/\tau_0)(\partial\tau/\partial t)_0$ following the proposal of Dalgleish (1983). However, since turbidity measurements will be used in this work to compare aggregation rates under different conditions, it is important to examine their dependence on the physical properties of the particles and the medium. The turbidity of a suspension of polydispersed spherical particles, sufficiently diluted so that multiple scattering can be neglected and randomly positioned, which implies the absence of particles interactions, can be expressed as (Kerker, 1969)

$$\tau = C_{sca} N \tag{2}$$

where C_{sca} is the particle cross section for scattering, which depends on the size of the particles and refractive indices of the particles and the medium. At initial times of aggregation, considering only the aggregating particles and their doublets (Russel et al., 1989)

$$\tau = (C_{sca})_1 N_1 + (C_{sca})_2 N_2 \tag{3}$$

where $(C_{csa})_1$ and $(C_{csa})_2$ are the scattering cross sections of primary aggregating particles and doublets, respectively, and N_1 and N_2 are the corresponding number densities.

The initial rate of turbidity change in this conditions can be related to the initial rate of doublet formation using eq 3, giving the following expression:

$$\frac{1}{\tau_0}\left(\frac{\partial\tau}{\partial t}\right)_0 = k_2\left[\frac{(C_{sca})_2}{2(C_{sca})_1} - 1\right]N_0 \tag{4}$$

Using the expressions developed by Wippler for multiplets of spheres (Kerker, 1969) whit application of the Rayleigh−Debye−Gans theory, it is possible to show that the cross section ratio is a function only of the radii of the primary particles. Therefore, comparison between aggregation rates of samples under different conditions must take into account that changes in $(1/\tau_0)(\partial\tau/\partial t)_0$ may involve changes in size of aggregating particles.

The kinetics of coagulation was studied following the turbidity of the system at 600 nm, as described in a previous work (Gatti et al., 1996). Milk samples equilibrated at the working temperature were diluted, and the dilutions, always maintained at the working temperature, were allowed to equilibrate to reach a constant turbidity value. An aliquot of these dilutions was poured into a 3 mL spectrophotometer cuvette in a jacketed cuvette holder, maintained at the working temperature by water circulation. Coagulation was started by rennet addition to the cuvette. The mixture was gently stirred with a Teflon stirrer for 5 s, and its absorbance at 600 nm was recorded as a function of time. The concentration of rennet used was sufficient to hydrolyze casein maximally in a short time at the start of the process, as was shown following the release of peptides soluble in 30 g/L trichloroacetic acid (Queiroz Macedo et al., 1993), by the Lowry−Peterson method (Peterson, 1977). The initial time of aggregation was taken as the time at which 100% of the CMP release was reached. $(1/\tau_0)(\partial\tau/\partial t)_0$ was measured as the maximal slope of a τ vs t plot after the initial time, divided by τ_0.

The state of CM and HCM was followed by the values of τ at 400 nm and $\alpha = -(\partial \log \tau/\partial \log \lambda)$ (Horne, 1986), which varies inversely to the particle radius. τ was measured as absorbance, and residual τ values due to fat globules yet present were measured after dissociation of CM and HCM by addition of a 0.11 M EDTA, 0.15 M NaOH, and 0.1 (v/v) Tween 20 solution and were subtracted from the τ values of the dilutions.

A Beckman DU 640 spectrophotometer was used for absorbance measurements, using rectangular optical glass cuvetts with frosted lateral walls. No corrections were introduced in τ measurements for light scattered at small angles in the forward direction, since the particles studied were of size comparable to the wavelength, a case in which only minute differences may be expected between τ and absorbance measurements (Van de Hulst, 1957).

RESULTS AND DISCUSSION

Figure 1 shows an example of the CMP release as a function of time for both CM and HCM, at given casein concentration and temperature. According to this plot, the time at which 100% of the CMP release has been reached was 13 s for CM and 15 s for HCM, both of which are shorter than the times at which turbidity

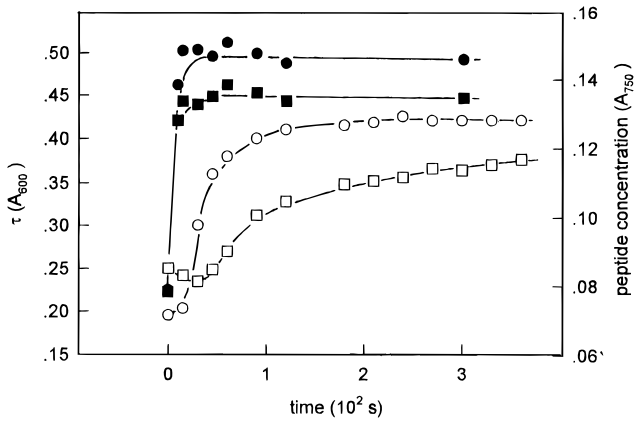

Figure 1. Time course for the release of casein macropeptides and the aggregation by rennet of both *p*CM and H*p*CM. Diluting buffer system was 5 mM $CaCl_2$ and 10 mM imidazole−HCl, pH 6.4. Casein concentration was 0.15 g/L. Concentrations of peptide fractions soluble in 30 g/L trichloroacetic acid measured by the Lowry−Peterson method as absorbance at 750 nm are illustrated for (●) *p*CM and (■) H*p*CM. Turbidity at 600 nm is illustrated for (○) *p*CM and (□) H*p*CM. Temperature was 308 K.

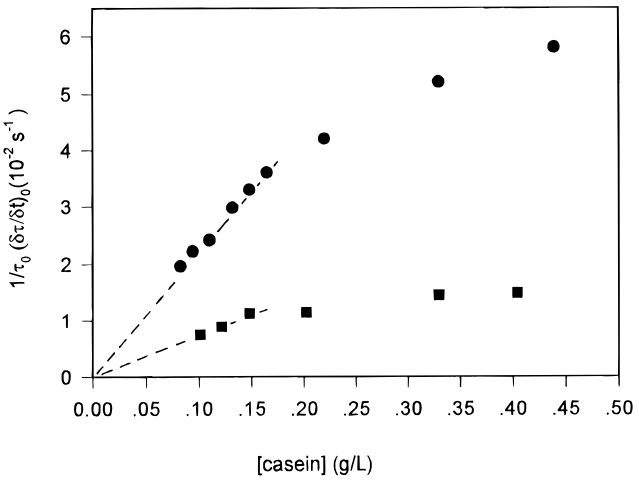

Figure 2. $1/\tau_0(\delta\tau/\delta t)_0$, rate of relative turbidity increase as a function of casein concentration for (●) *p*CM and (■) H*p*CM. Buffer was 5 mM $CaCl_2$ and 10 mM imidazole−HCl, pH 6.4. Temperature was 308 K. Each point is the mean of at least five determinations.

starts to increase in each case. Similar plots were obtained for the different casein concentrations used by maintaining the same enzyme/substrate ratio, determining the times of 100% CMP release, and measuring $(\partial\tau/\partial t)_0$ as explained under Materials and Methods. When different temperatures or different glycerol concentrations were used, at constant casein concentration, the times of 100% CMP release were determined at least for the higher and lower values of the variable under study for CM and HCM. These values were applied to the measure of $(\partial\tau/\partial t)_0$ in each case, as explained above.

Effect of *p*CM and H*p*CM Concentration. τ values of both CM and HCM suspensions in the range of 0.08−0.44 g/L showed lineal behavior, while α values remained practically constant for both kinds of suspensions in the same casein concentration range. These results indicated that neither dissociation nor aggregation processes are present in this concentration range.

Comparison of the τ and α values obtained for the two kinds of samples showed that τ_0 at 400 nm for HCM was higher than the value for CM at the same casein concentration, with lower values of α. These results are coherent both with an increase of average size of CM by high heating, as observed by electron microscopy (Mohammad and Fox, 1987), and with a variation of MC refractive index because of calcium phosphate precipitation on the micelles.

However, estimations of $(C_{sca})_1/(C_{sca})_2$ using theoretical developments previously described (Gatti et al., 1996) showed that the differences between the values of the scattering cross-section ratio for *p*CM and H*p*CM can be neglected. This enables us to considered $(1/\tau_0)$-$(\partial\tau/\partial t)_0$ as proportional to k_2.

Figure 2 shows the values of $(1/\tau_0)(\partial\tau/\partial t)_0$ as a function of casein concentration, for both *p*CM and H*p*CM. The results showed that the aggregation rate for H*p*CM was lower than for *p*CM in the entire range of casein concentration. An initial lineal behavior was observed for both *p*CM and H*p*CM. In the first case, these results confirmed previous observations in the sense that *p*CM aggregation is initially a second-order process, according to Smoluchowski's theory of Brownian aggregation (Gatti et al., 1996). It was also the case for H*p*CM aggregation, showing that the changes introduced in CM structure did not affect the mechanism of the process. At casein concentrations higher than these values, deviations from linearity were evident. Since the hypothesis of initial doublet formation in Brownian flocculation has been proposed for dilute homogeneous systems, the deviations observed could be setting the limit for the application of this approach to our systems. However, it must be taken into account that these deviations could also imply limitations in the precise measurement of $(\partial\tau/\partial t)_0$ for higher initial rates of flocculation. Therefore, the results obtained showed that the hypothesis proposing an initial second-order kinetics can be considered supported for sufficiently dilute milk dilutions with the method used.

Heating of milk in the conditions described in this work could be expected to produce denaturation of whey proteins, formation of complexes of these denatured proteins with κ-casein, with release of a fraction of κ-casein (Law, 1996; Leaver et al., 1995; Calvo, 1995; Lucey et al., 1993), and precipitation of calcium phosphate (Jeurnick, 1992). None of these processes could be the cause of an important change in the net charge of the micelles. It seems then logical to attribute the high increase of stability observed for H*p*CM mainly to an increment of steric stability.

Effect of Temperature. Temperature increase produced increment in τ for both CM and HCM, an increase that could be attributed to a micellar voluminosity decrease (Penders and Vrij, 1990). A slight increase of α was coherently detected in both cases. The small value of the α variation, on the order of 1.2% for both CM and HCM, enabled us to assume that the variation in the micelle size produced by the temperature increase used gave negligible differences in the scattering cross-section ratio $(C_{sca})_1/(C_{sca})_2$. In these conditions, the values of $(1/\tau_0)(\partial\tau/\partial t)_0$ were used as proportional to k_2 and plotted in Figure 3 as function of temperature, for the aggregation of both *p*CM and H*p*CM.

Although the initial rate of aggregation was higher for *p*CM in the entire range of temperatures studied, the curves in Figure 3 presented similar characteristics. An extrapolation of the experimental values to low temperatures showed that both of the aggregation rates tended to be null in the 10−15 °C range. This is a well-documented behavior of *p*CM suspensions, known as the Berridge effect (Walstra, 1990), which appeared here in a similar way for H*p*CM suspensions. The data

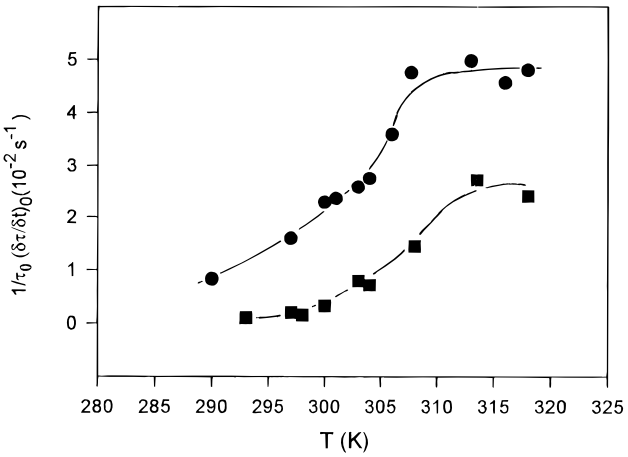

Figure 3. $1/\tau_0(\delta\tau/\delta t)_0$, rate of relative turbidity increase as function of temperature for (●) *p*CM and (■) H*p*CM. Buffer was 5 mM $CaCl_2$ and 10 mM imidazole−HCl, pH 6.4. Casein concentration was 0.30 g/L. Each point is the mean of at least five determinations

published on *p*CM ζ-potential (Carlson et al., 1987) at different temperatures showed that the *p*CM high stability below 10 °C could not be attributed to an increment of electrostatic repulsion at such low temperatures. This phenomenon could better be related to the presence of remaining steric stability after the proteolytic cleavage of the hydrophilic moiety of κ-casein (Dalgleish, 1983; Gatti et al., 1996). From this approach, the minimal temperature at which *p*CM or H*p*CM aggregation occurs could be interpreted as an upper critical flocculation temperature (UCFT). Such a critical temperature appears in colloidal dispersions of particles stabilized by an external layer of water-soluble polymers in aqueous media and is usually associated with a steric stability of enthalphic nature (Hunter, 1995). In these cases, the predominant factor contributing to the steric stability is the positive enthalpic component of the contact dissimilarity term in the free energy of interpenetration of two particle external layers, that is to say, the enthalpic component of the interaction parameter χ_1 of the Flory−Huggins theory of polymer solutions, applied to the interaction of the stabilizing surface polymers and the dispersion medium. This positive term, a consequence of specific interactions (such as hydrogen bonding) of water molecules with suitably polar stabilizing moieties, contributes to make positive the free energy of flocculation, giving stability to the suspension. Nevertheless, in the case of both *p*CM and H*p*CM, an external layer expansion can be expected also at low temperatures, similar to that observed for whole CM (Walstra, 1990). This expansion has been attributed to the protrusion of β-casein chains because of the weakening, at low temperatures, of the hydrophobic bonds that retain this casein in the micellar structure.

The increase of temperature above 15 °C produced a loss of stability in both *p*CM and H*p*CM suspensions, leading to coagulation, with a progressive increase of the initial rate of aggregation. This behavior could be the result of both the decrease of the enthalpic component of the contact dissimilarity term, with simultaneous absolute value increment of negative entropic contributions that promote flocculation (Hunter, 1995), and the contraction of the stabilizing external layer by retraction of the β-casein chains because of the progressive strengthening of their hydrophobic bonds. Both effects seemed to tend to saturation for temperatures above 42 °C. However, the maximal aggregation rates reached are yet well below the values expected for Smoluchowski's rapid coagulation (Dalgleish, 1983), indicating the permanence of some steric stability even at high temperatures.

It is interesting to note that, although the curves for *p*CM and H*p*CM in Figure 3 exhibited similar shapes, suggesting that the effect of temperature on stability was similar for both suspensions, the values of initial rate of aggregation were always lower for H*p*CM in the entire range of temperatures studied. Since the effects of heating (calcium phosphate precipitation and β-lg−κ-casein complex formation) do not introduce important charge variations, the higher stability shown by H*p*CM could not be attributed to an increase of electrostatic repulsion between micelles. It could be most likely attributed to structural changes in HCM surface leading to a higher steric stability. In fact, electron microscopy observations have detected a ragged surface of appendages protruding from HCM (Mohammad and Fox, 1987), structural features that may be probably involved in an increase of steric stability of H*p*CM compared with *p*CM.

Effect of Medium Viscosity. Both τ and α slightly increased upon addition of glycerol to the medium up to 5%. These initial variations were followed by a decrease of turbidity of both CM and HCM with higher glycerol concentrations, a decrease that could be attributed to the increase in refraction index of the media. Although α values showed also a slight decrease (<2%) for increasing glycerol concentrations above 5%, suggesting a slight swelling of CM and HCM, these variations can be considered as too low to introduce significative changes in the ratio of scattering cross section, enabling us to use the values of $(1/\tau_0)(\partial\tau/\partial t)_0$ as directly proportional to k_2.

The initial effect described for glycerol concentrations up to 5% was higher for CM than for HCM and more pronounced at low temperature. This observation could indicate the presence of an interaction of glycerol with the casein micelles, producing a slight compaction of the particles.

$(1/\tau_0)(\partial\tau/\partial t)_0$ values were plotted as function of reciprocal viscosity in Figure 4 to examine the effect of medium viscosity on the aggregation rate. This plot showed that an initial addition of glycerol to the medium increased the initial aggregation rate both for *p*CM and H*p*CM. Further addition of glycerol produced gradual decrease of both aggregation rates, reaching at high glycerol concentration values lower than the values estimated in the absence of glycerol. The aggregation rate for slow Brownian aggregation can be expressed as (Smoluchowski)

$$k_2 = \frac{8kT}{3\eta W} = \frac{k_2^{\,0}}{W} \qquad (5)$$

where k is the Boltzmann constant, η is the viscosity of the medium, W is the stability factor (ratio of the particle collision frequency to the frequency with which collisions result in aggregation), and $k_2^{\,0}$ is the kinetic constant when all of the collisions lead to lasting contacts. If the variations observed were only due to viscosity changes of the medium, a lineal increase of $(1/\tau_0)(\partial\tau/\partial t)_0$ for increasing $1/\eta$ would be expected, instead of the curves obtained in Figure 4. The shape of these curves suggests that glycerol produced another effect on *p*CM and H*p*CM stability, besides the aggregation

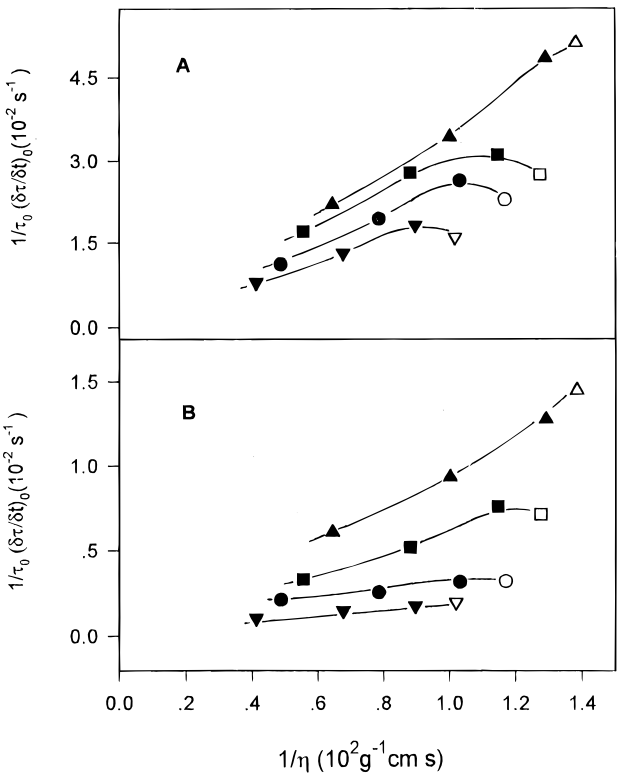

Figure 4. $1/\tau_0(\delta\tau/\delta t)_0$, rate of relative turbidity increase as function of reciprocal viscosity for (A) *p*CM and (B) H*p*CM. Buffer was 5 mM $CaCl_2$ and 10 mM imidazole−HCl, pH 6.4. Temperatures were (▽, ▼) 297 K, (○, ●) 300 K, (□, ■) 304 K, and (△, ▲) 313 K. Hollow symbols represent media without glycerol. Solid symbols represent media with addition of appropriate glycerol concentrations. Casein concentration was 0.30 g/L. Each point is the mean of at least five determinations.

rate decrease due to the medium viscosity increase. This effect appears to involve an initial interaction of glycerol with casein micelles which produced a decrease of micelles size, as suggested by τ and α variations, accompanied by a decrease of stability at low glycerol concentration, similar to the action of ethanol on CM stability described by Horne (1985, 1987). This effect appeared as higher at low temperatures, a fact that could suggest the presence of electrostatic interactions between glycerol and the micelle surface. These interactions seemed to have been produced in higher extension for the *p*CM surface polymeric chains than for the H*p*CM ones. In fact, glycerol can be attracted by residues containing carboxyl, amino, or hydroxyl groups and become bound to the protein surface through formation of multiple hydrogen bonding, replacing in this way structured water bound to the protein (Na, 1986). These actions may likely result in structural changes in the protein chains involved in the external layer of the particles, thereby changing steric stability. These structural changes could even include variations of the micellar to serum protein ratio, by glycerol promotion of dissociation or association process. When the interaction of glycerol with micelles becomes saturated, the action of higher concentrations of glycerol produces a reduction of the aggregation rate by the increase of the medium viscosity, and the aggregation rate varies as expected according to eq 5.

Conclusions. The experimental data here analyzed suggested that the stability of both *p*CM and H*p*CM suspensions involves an important contribution of steric stability, which could be the predominant factor in the aggregation behavior of such particles. This component could be mainly provided by β-casein and appears as enthalpic in nature at low temperatures. Its presence could explain the Berridge effect, present in both kinds of suspensions, as the existence of an UCFT. The results of aggregation kinetics for *p*CM and H*p*CM can be well interpreted considering these aggregation processes as cases of slow Brownian flocculation, according to the Smoluchowski theory. The separation of the first and second stages of enzymic coagulation allows us to confirm that heating of milk at temperatures high enough to denature whey proteins produced a decrease of the aggregation rate. This effect could be mainly attributed to an increment of the steric stability of casein micelles related to changes in their surface structure.

LITERATURE CITED

Calvo, M. M.; Law, A. J. R.; Leaver, J. Heat-induced interactions between serum albumin, immunoglobulin, and κ-casein inhibit the primary phase of renneting. *J. Agric. Food Chem.* **1995**, *43*, 2823−2827.

Carlson, A.; Hill, C.; Olson, N. Kinetics of milk coagulation: II. Kinetics of secondary phase: micelle flocculation. *Biotechnol. Bioeng.* **1987**, *29*, 590−600.

Dalgleish, D. G. Proteolysis and aggregation of casein micelles treated with immobilized or soluble chymosin. *J. Dairy Res.* **1979**, *46*, 653−661.

Dalgleish, D. G. Coagulation of renneted bovine casein micelles: dependence on temperature, calcium ion concentration and ionic strength. *J. Dairy Res.* **1983**, *50*, 331−340.

Dalgleish, D. G. The effect of denaturation of β-lactoglobulin on renneting-a quantitative study. *Milchwissenschaft* **1990**, *45*, 491.

Darling, D. F.; Van Hooydonk, A. C. M. Derivation of a mathematical model for the mechanism of casein micelle coagulation by rennet. *J. Dairy Res.* **1981**, *48*, 189−200.

Dzurec, D. J.; Zall, R. R. Effect of heating, cooling and storing milk on casein and whey protein. *J. Dairy Sci.* **1985**, *68*, 273−280.

Gatti, C. A.; Pires, M. S. Effect of monovalent cations on the kinetics of renneted milk coagulation. *J. Dairy Res.* **1995**, *62*, 667−672.

Gatti, C. A.; Risso, P.; Pires, M. Spectrofluorometric study on surface hydrophobicity of bovine casein micelles in suspension and during enzymic coagulation. *J. Agric. Food Chem.* **1995**, *43*, 2339−2344.

Gatti, C. A.; Pires, M. S.; Orellana, G. A.; Pereyra, J. Kinetic evidence for steric destabilization of casein by rennet enzyme activity. *J. Chem. Soc., Faraday Trans.* **1996**, *92*, 2575−2578.

Green, M. L.; Morant, S. V. Mechanism of aggregation of casein micelles in rennet-treated milk. *J. Dairy Res.* **1981**, *48*, 57−63.

Horne, D. S. Steric stabilization and casein micelle stability. *J. Colloid Interface Sci.* **1986**, *111*, 250−260.

Hunter, R. J. *Foundations of Colloid Science*; Clarendon Press: Oxford, U.K., 1995; pp 450−493.

Jeurnick, Th. J. M. Changes in milk on mild heating:turbidity measurements. *Netherlands Milk Dairy J.* **1992**, *46*, 183−196.

Kerker, M. *The Scattering of Light and Other Electromagnetic Radiation*; Academic Press: New York, 1969; pp 417, 446, 428.

Kuaye, A. Y. An ultraviolet spectrophotometric method to determine milk protein content in alkaline medium. *Food Chem.* **1994**, *49*, 207−211.

Law, A. J. R. Effects of heat treatment and acidification on the dissociation of bovine casein micelles. *J. Dairy Res.* **1996**, *63*, 35−48.

Leaver, J.; Law, A. J. R.; Horne, D. S.; Banks, J. M. Influence of heating regime and pH on the primary phase of renneting of whole milk. *Int. Dairy J.* **1995**, *5*, 129−140.

Lucey, J. A.; Gorry, C.; Fox, P. F. Rennet coagulation properties of heated milk. *Agric. Sci. Finl.* **1993**, *2*, 361−368.

Mohammad, K. S.; Fox, P. F. Heat induced microstructural changes in casein micelles before and after heat coagulation. *N. Z. J. Dairy Sci. Technol.* **1987**, *22*, 191.

Na, G. C. Interaction of calf skin collagen with glycerol: linked function analysis. *Biochemistry* **1986**, *25*, 967−973.

Pappas, C. P. Interactions between milk proteins: Influence of heat, calcium and lactose. I—Interactions between whole casein and β-lactoglobulin and κ-casein and β-lactoglobulin. *Lebensm. Wiss. Technol.* **1992**, *25*, 102−112.

Payens, T. A. J. Casein micelles: the colloid-chemical approach. *J. Dairy Res.* **1979**, *46*, 291−306.

Penders, M. H. G. M.; Vrij, A. A turbidity study on colloidal silica particles in concentrated suspensions using the polydisperse adhesive hard sphere model. *J. Chem. Phys.* **1990**, *93* (5), 3704−3711.

Peterson, G. L. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem.* **1977**, *83*, 346−356.

Queiroz Macedo, I.; Faro, C. J.; Pires, E. M. Specificity and kinetics of the milk-clotting enzyme from Cardoon (*Cynara carduncul*us L.) toward bovine κ-casein. *J. Agric. Food Chem.* **1993**, *41*, 1537−1540.

Reddy, I. M.; Kinsella, J. E. Interaction of β-lactoglobulin with κ-casein in micelles as assessed by chymosin hydrolysis: effect of temperature, heating time, β-lactoglobulin concentration and pH. *J. Agric. Food Chem.* **1990**, *38*, 50−58.

Russel, W. B.; Saville, D. A.; Schowalter, W. R. *Colloidal Dispersions*; Cambridge University Press: Cambridge, U.K., 1991; p 275.

Van de Hulst, H. C. *Light Scattering by Small Particles*; Wiley: New York, 1957; p 729.

Van Hooydonk, A. C. M.; Walstra, P. Interpretation of the kinetics of the renneting reaction in milk. *Netherlands Milk Dairy J.* **1987**, *41*, 19−47.

Van Hooydonk, A. C.; de Koster, M. P. G.; Boerrigter, I. J. The renneting properties of heated milk. *Netherlands Milk Dairy J.* **1987**, *41*, 3−18.

Walstra, P. On the stability of casein micelles. *J. Dairy Sci.* **1990**, *73*, 1965−1979.

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