Kinetics of Casein Micelle Destabilization*

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Destabilization of casein micelles in reconstituted skim milk was studied experimentally in both nonmixed and mixed conditions. The process of micelle destabilization was described with a three-step kinetic model accounting for hydrolysis of casein in both stable and partially destabilized micelles. This model was used for the estimation of kinetic parameters of κ-casein hydrolysis proceeding on the surface of casein micelles and kinetic parameters of micelle destabilization. The influence of various process variables, such as mixing and enzyme concentration, on the hydrolysis of κ-casein and destabilization of casein micelles was evaluated. The presented model provides a good description of casein micelle destabilization within a specified range of process variables.

Caseins constitute approximately 80 % of bovine milk proteins. The majority of the casein proteins exists as large colloidal agglomerates, called casein micelles, which also contain mineral constituents, especially calcium phosphate. The casein micelles are suspended in the milk serum. Several experimental techniques have been developed to examine the structure and properties of these micelles. *Hansen et al.* [1] studied the structure of casein micelles by small-angle neutron scattering and static light scattering. They analyzed the experimental data assuming that the casein micelle consists of smaller spherical units, called submicelles. Regions of amorphous calcium phosphate link the submicelles to each other. The average micellar radius is about 100—120 nm and the submicellar radius is about 7 nm, both with the polydispersity of about 40—50 %. Micelles are fairly voluminous, containing large amount of solution similar to the milk serum.

The principal casein fractions are α-casein, β-casein, and κ-casein. Caseins are conjugated proteins with phosphate groups esterified to serine residues. Phosphate groups are important to casein association and the structure of casein micelle. Calcium binding by individual caseins is proportional to phosphate content. Physicochemical measurements on the native bovine casein micelle have indicated that the outermost region comprises a diffuse layer of flexible hydrophilic polypeptide chains from the C-terminal half of κ-casein. Flexible polypeptide chains, known as the hairy layer, are projected into the solution from the surface of the micelles and present a virtually inpenetrable barrier against aggregation, so that casein micelles are stabilized sterically [2—4]. In addition, micelles also carry negative charge on their surfaces [4] although it is believed that the primary stabilization mechanism is sterical [3, 4].

Native casein micelles thus form a stable colloidal dispersion, which can be destabilized *via* hydrolysis of κ-casein by a proteolytic enzyme (rennet). Casein micelles destabilized by renneting then undergo spontaneous aggregation. Experimental techniques monitoring this aggregation include turbidity measurements [5, 6] and light scattering [7, 8] of diluted casein micelle suspensions, and clotting of the destabilized micelles in the nondiluted reaction mixture by the acetate buffer at pH 5.2 and temperature 5 °C [9, 10]. *Reuttimann* and *Ladish* [11] also used the dark-field illumination microscopy for the observation of the particle size distribution and the aggregate conformation of casein solutions in the native environment.

It is well known that here is a lag time after the enzyme addition and before any structural changes in the casein solution are observed. Before the enzyme addition as well as in an early part of the lag period, the native casein micelles are constantly moving about

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due to Brownian motion and exist as a stable dispersion of single particles. The delay between the initiation of the enzyme reaction and the visually observable coagulation is believed to be due to time required for the enzyme to produce a significant concentration of destabilized micelles, which can undergo diffusion-controlled aggregation [12].

In the renneting process a small soluble peptide corresponding to the hydrophilic part of κ-casein molecules, glycomacropeptide, is released from the micelles by a proteolytic enzyme. The hydrophobic part of the κ-casein molecule, *para*-κ-casein, remains associated with the micelle. The hydrolysis kinetics of individual κ-casein molecules is usually described using one-step or two-step kinetic schemes, where the first step is always the first order with respect to κ-casein [6, 13]. The hydrolysis of κ-casein molecules occurs in a random fashion over the surface micelles resulting in thinning of the diffuse layer of hydrophilic polypeptide chains, so that the steric repulsion between micelles is gradually diminished. As a result of shearing off κ-casein hairs, the micelle radius decreases and approximately half of the micelle surface charge is lost. Because of the extensive hydration of the micelle surface layers, the true decrease in the micelle radius may be as much as 12 nm [14]. The decrease in the micellar radius is consistent with the loss of the hairy macropeptide layer of the particles [15]. It was also found that the electrical conductivity of milk decreased as viscosity increased during the renneting process. The conductivity change was interpreted by *Dejmek* [16] as a change in the way casein micelles obstructed the path of the charge-carrying ions.

Hydrolysis of κ-casein results in unstable aggregating micelles. The aggregation rate becomes significant only when a large majority of surface κ-casein molecules is hydrolyzed. According to *Dalgleish* [17] as much as 97 % of κ-casein molecules at the micelle surface must hydrolyze before the micelle can aggregate. Since the number of κ-casein molecules per micelle is relatively small, the process of κ-casein hydrolysis results in nonuniform distribution of hydrolyzed κ-casein molecules among individual micelles. Various micelles become unstable at various times and their subsequent aggregation happens gradually. The kinetics of κ-casein hydrolysis is thus coupled with the kinetics of micelle aggregation, which depends on the distribution of hydrolyzed κ-casein molecules within the ensemble of micelles.

In our previous work [18] we modeled the process of κ-casein micelle destabilization by coupling kinetic equations of κ-casein hydrolysis with a probabilistic model for distribution of hydrolyzed κ-casein molecules. Since it is not possible to reliably determine this distribution with current experimental techniques, it was necessary to build assumptions about this distribution into a model. The model was then validated by matching experimental data on kinetics of micelle destabilization against the model, while simultaneously fitting rate constants of κ-casein hydrolysis kinetics and the critical degree of hydrolysis in the probabilistic model of micelle destabilization. In this work we introduce a new approach allowing us to model the process of micelle destabilization on purely kinetic basis.

THEORETICAL

The process of micelle destabilization begins with proteolytic hydrolysis of κ-casein to *para*-κ-casein (*i.e.* the hydrophobic part of κ-casein) and glycomacropeptide. The *para*-κ-casein remains tied by strong protein—protein interactions to the rest of the casein micelle. The aggregation properties of casein micelles are altered gradually, since a micelle is capable of aggregation only after a critical proportion of its κ-casein molecules is hydrolyzed. While this critical proportion has not been reached, the hydrolysis product (*para*-κ-casein) is associated together with the substrate (κ-casein) in a stable micelle. This micelle can thus be thought of as a complex of substrate and product molecules. As hydrolysis continues, the number of product molecules in this complex increases and the number of substrate molecules decreases, while their total amount remains constant. Detailed modeling of this process is complicated and leads to introduction of various assumptions with additional fitting parameters. In the model introduced here we choose to approximate the micelle destabilization process by an effective association between the substrate and the product, which are assumed to form a simple 1 : 1 complex. This complex represents the state of casein (both κ-casein and *para*-κ-casein) in micelles that are still stable and thus not able to aggregate. When a sufficient amount of κ-casein is hydrolyzed at the micelle surface, the micelle becomes unstable and the product is released from the complex. The experimentally measured amount of casein in destabilized micelles can thus be identified with the product.

Enzyme-catalyzed hydrolysis of κ-casein is described by the same mechanism regardless of whether it is associated with the *para*-κ-casein in the complex or not. Since we are always concerned with the hydrolysis of κ-casein molecules at the surface of a micelle, it is appropriate to use the same rate constants for either case. The rate constant of the substrate—product complex formation is related to the fraction of hydrolyzed κ-casein molecules needed for micelle destabilization. A small rate constant value corresponds to a small fraction of hydrolyzed molecules needed for destabilization. A large rate constant value corresponds to a situation when destabilization happens in later stages of the hydrolysis process when a large percentage of κ-casein molecules have been hydrolyzed. Thus the rate constant of complex formation is a single empirical parameter describing micelle destabiliza-

tion process coupled to the proteolytic hydrolysis kinetics of κ-casein. Rate constants for both proteolytic hydrolysis and substrate—product complex formation are determined by fitting the measured time dependence of the fraction of destabilized micelles, which is identified with the relative amount of product as defined in the model.

We now formally introduce the model using the following notation: the hydrolysis substrate (denoted S) is κ-casein at the surface of micelles, the hydrolysis product (denoted P) is *para*-κ-casein, the hydrolysis side product is glycomacropeptide (denoted G), and κ-casein with *para*-κ-casein form the substrate—product complex (denoted C). In the presence of a proteolytic enzyme E, the substrate S is hydrolyzed to the product P and the side product G. Assuming a two-step irreversible mechanism of enzyme-catalyzed hydrolysis, we can write the following reactions

$$\mathrm{S} + \mathrm{E} \xrightarrow{k_1} \mathrm{SE} \xrightarrow{k_2} \mathrm{P} + \mathrm{G} + \mathrm{E} \qquad (A)$$

However, *para*-κ-casein remains associated together with the unhydrolyzed κ-casein in micelles. As discussed above in the model description, we suppose they form the substrate—product complex C

$$\mathrm{P} + \mathrm{S} \xrightarrow{k_3} \mathrm{C} \qquad (B)$$

Unhydrolyzed κ-casein in this complex undergoes hydrolysis regardless of its companions in the micelle

$$\mathrm{C} + \mathrm{E} \xrightarrow{k_1} \mathrm{CE} \xrightarrow{k_2} 2\mathrm{P} + \mathrm{G} + \mathrm{E} \qquad (C)$$

The concentrations of the species S, E, P, C, CE, SE are denoted as *s, e, p, c, n, l*, respectively.

The initial concentrations of these species are

$$s = s_0 \quad e = e_0 \quad p = 0 \quad c = 0 \quad n = 0 \quad l = 0 \qquad (1)$$

Rate equations for the species concentrations are described by the mass action kinetic laws corresponding to the apparent elementary steps in the reaction schemes (A—C), together with the mass balance equations for the substrate and the enzyme. The dimensionless concentrations x_1, x_2, x_3, x_4 are defined by the equations

$$x_1 = \frac{s}{s_0} \quad x_2 = \frac{p}{s_0} \quad x_3 = \frac{e}{e_0} \quad x_4 = \frac{c}{s_0} \qquad (2)$$

The kinetic parameters are defined as

$$A = k_1 e_0 \quad B = k_2 \frac{e_0}{s_0} \quad C = k_3 s_0 \quad \varepsilon = \frac{e_0}{s_0} \qquad (3)$$

After substituting eqns (*2*) and (*3*) and the mass balance equations into the mass action kinetic equations, we get the following set of equations

$$\dot{x}_1 = -Ax_1x_3 - Cx_1x_2 \qquad (4)$$

$$\dot{x}_4 = Cx_1x_2 - Ax_3x_4 \qquad (5)$$

$$\varepsilon\dot{x}_3 = -Ax_1x_3 + B(1 - x_3) - Ax_3x_4 \qquad (6)$$

$$\varepsilon\dot{x}_2 = -Cx_1x_2\varepsilon + B(1 - x_1 - x_2 - 2x_4) \qquad (7)$$

Supposing $e_0/s_0 = \varepsilon \ll 1$, which is appropriate for our system, we can neglect terms multiplied by ε in eqns (*6*) and (*7*). We then obtain a simplified model containing two differential equations (*4*, *5*) and two algebraic equations

$$-Ax_1x_3 + B(1 - x_3) - Ax_3x_4 = 0 \qquad (8)$$

$$1 - x_1 - x_2 - 2x_4 = 0 \qquad (9)$$

The parameters *A, B,* and *C* were evaluated by fitting the experimental data for the fraction of the destabilized micelles, which is identified with the relative concentration of the product $x_2 = f(t)$. Numerical integration of eqns (*4, 5, 8, 9*) was performed using the 4th-order Runge—Kutta—Merson method. Rosenbrock optimization method was used for the fitting [19].

EXPERIMENTAL

The reconstituted skim milk solutions (powder concentration 0.1 g cm^{-3}) were prepared using nonfat dry skim milk Laktino (Promil-PML Nový Bydžov, Czech Republic) and 0.01 M-$CaCl_2$. A commercial rennet Hannilase powder (Chr. Hansen's Lab. Denmark A/S Copenhagen) was used.

The initial substrate concentration s_0 is the κ-casein concentration at the surface of micelles in the reconstituted milk. The numerical value of this quantity can only be assessed with considerable uncertainty, but it is not necessary to involve absolute concentration dimensions in our analysis. Instead, we chose the initial substrate concentration in the reconstituted skim milk solutions used here as the dimensionless unit $s_0 = 1$ for the substrate concentration. In order to obtain the relative enzyme concentration x_3 we chose as the dimensionless unit $e_0 = 1$ such a rennet solution concentration in phosphate buffer of pH 6.2, which was able to hydrolyze 0.1 g cm^{-3} nonfat reconstituted milk to the state at which the clot of casein micelles became visible in 40 min. We would like to stress that all concentrations reported in this paper and used in data analysis and evaluation of rate constants are dimensionless.

Native casein micelles with hydrophilic chains of glycomacropeptide are very stable at natural milk conditions. By removing glycomacropeptide chains, the properties of micelles are being changed. Destabilized micelles may fuse into bigger particles, flocculate and aggregate. The rate of these processes depends on con-

ditions, as temperature, enzyme and substrate concentrations, hydrodynamic conditions in the reactor, pH and the ionic strength of the reaction mixture. Amount of the destabilized micelles was determined by clotting by acetate buffer at pH 5.2 and temperature 5 °C [9, 10].

In our experiments we equilibrated 50 cm^3 of reconstituted milk in a (mixed or nonmixed) batch reactor for 30 min at the reaction temperature of 32 °C before any enzymatic reaction was started. The magnetic stirrer frequency used in the mixed reactor was 450 min^{-1}. To start the reaction, the enzyme was injected into the milk solution. At predetermined reaction times the enzymatic reaction was stopped by adding 1 M-NaOH. Then, pH was adjusted to 8.2 and the reaction mixture was cooled to 5 °C. 1 cm^3 of acetate buffer at 5 °C was added to 1 cm^3 of the reaction mixture. This solution with pH 5.2 was centrifuged for 15 min at a temperature of 5 °C. Clotted casein micelles were separated, dissolved in 15 cm^3 of 1 M-NaOH and 25 cm^3 of water was added. The protein was determined by ultraviolet absorption at 290 nm [13, 14]. The relative concentration of clotted micelles x_2 was determined as the ratio of the absorbance at the specific time t to its equilibrium value at long reaction times.

RESULTS AND DISCUSSION

Experimental kinetic data collected in this study were the time dependences of the relative concentrations of destabilized casein micelles x_2 for four different initial enzyme concentrations e_0: 2, 2.4, 3, 4. Data for nonmixed and mixed batch reactors are shown in Figs. 1 and 2, respectively. The shape of the plots of the relative concentration of destabilized micelles *vs.* time in these figures is similar to the time evolution of the turbidity of renneted micelles [6]. Three distinct stages of the micelle destabilization were observed. During the first stage, known as the lag time, little or none tendency for micelle flocculation exists, *i.e.* the micelles are predominantly stable. This is consistent with previous observations using the dark-field illumination microscopy [11, 20]. Immediately after the addition of the rennet, the system consisted mainly of single micelles and their small clusters. The second stage represents a rapid increase in the concentration of destabilized micelles. Successful collisions between destabilized micelles result in permanent contacts between particles or clusters. As the enzymatic hydrolysis and destabilization of micelles proceed, the clusters grow [10]. During the third stage, the relative concentration of destabilized casein micelles grows very slowly, since the extent of κ-casein hydrolysis is already large, while aggregated clusters are reaching a limiting size. These three stages are clearly distinguished on the curves plotted in Figs. 1 and 2.

Experimental data $x_2 = f(t)$ were used in the parameter-fitting procedure. Optimal values of the parameters *A, B, C* and the corresponding residual sum squared $\sum(x_2 - x_{2,\mathrm{calc}})^2$ for the nonmixed batch reactor are given in Table 1. Solid lines in Fig. 1 show time evolution of relative product concentration calculated using eqns (*4, 5, 8, 9*) with optimal kinetic parameters from fitting experimental data using our model. The hydrolysis rate constants k_1, k_2 and the rate constant for the complex formation k_3 were evaluated from eqn (*3*) with the corresponding enzyme concentrations for the initial substrate concentration $s_0 = 1$. Calculated values of k_1, k_2, and k_3 are listed

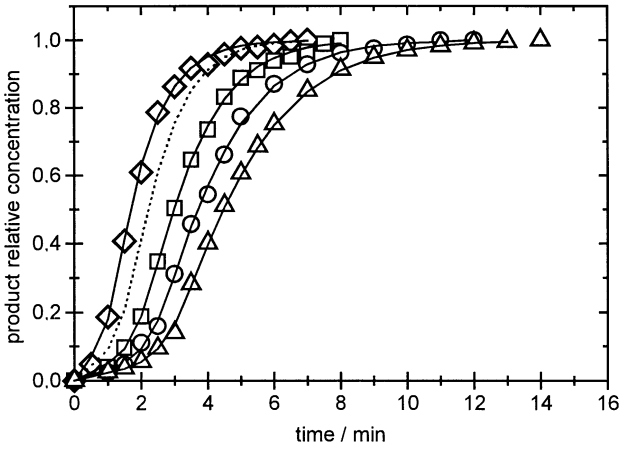

Fig. 1. Measured relative concentrations of destabilized casein micelles x_2 in dependence on time in a nonmixed batch reactor. The points for e_0: ◊ 4, □ 3, ○ 2.4, △ 2. Solid lines – data calculated from eqn (*11*) with optimal kinetic parameters. Dashed line – data calculated for $e_0 = 4$ with average kinetic parameters of the model kinetics calculated for the three other enzyme concentrations.

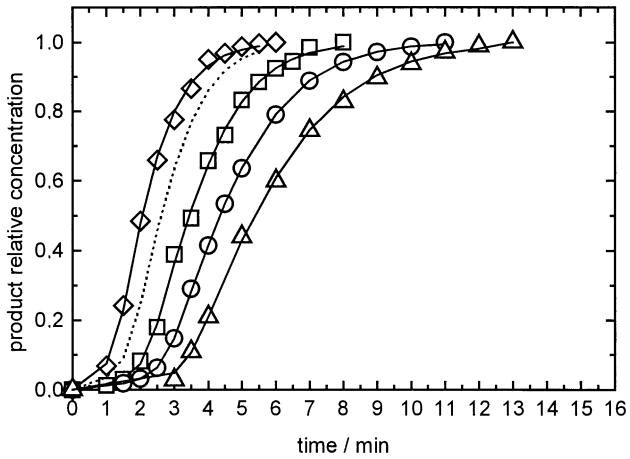

Fig. 2. Measured relative concentrations of destabilized casein micelles x_2 in dependence on time in a mixed batch reactor. The points for e_0: ◊ 4, □ 3, ○ 2.4, △ 2. Solid lines – data calculated from eqn (*11*) with optimal kinetic parameters. Dashed line – data calculated for $e_0 = 4$ with average kinetic parameters of the model kinetics calculated for the three other enzyme concentrations.

Table 1. Estimated Kinetic Parameters for a Nonmixed Batch Reactor

e_0	A	B	C	$\sum(x_2 - x_{2,\mathrm{calc}})^2$	k_1	k_2	k_3
	min^{-1}	min^{-1}	min^{-1}		min^{-1}	min^{-1}	min^{-1}
4	1.045	2.084	19.2	0.001775	0.261	0.521	19.2
3	0.856	0.552	15.7	0.001052	0.285	0.184	15.7
2.4	0.696	0.437	16.8	0.004170	0.290	0.182	16.8
2	0.589	0.359	16.5	0.001086	0.295	0.180	16.5

Table 2. Estimated Kinetic Parameters for a Mixed Batch Reactor

e_0	A	B	C	$\sum(x_2 - x_{2,\mathrm{calc}})^2$	k_1	k_2	k_3
	min^{-1}	min^{-1}	min^{-1}		min^{-1}	min^{-1}	min^{-1}
4	1.174	0.859	30.0	0.002885	0.294	0.215	30.0
3	0.941	0.381	35.8	0.002452	0.314	0.127	35.8
2.4	0.747	0.301	33.8	0.000113	0.311	0.125	33.8
2	0.586	0.250	36.5	0.008716	0.293	0.125	36.5

in Table 1. The calculated kinetic constants are in a good mutual agreement for the relative enzyme concentration between 2 and 3.

Corresponding results were also obtained for the mixed batch reactor. Optimal values of parameters *A, B, C,* the residual sum squared, and the calculated values of the rate constants k_1, k_2, and k_3 are given in Table 2. Solid lines in Fig. 2 represent the relative product concentration calculated from eqns (*4, 5, 8, 9*) using the optimal values of parameters. As for the nonmixed batch reactor, the calculated kinetic constants are in a good mutual agreement for the relative enzyme concentrations 2—3.

In both mixed and nonmixed case, the optimal rate constants obtained by fitting the experimental data for the relative enzyme concentration 4 are different from those for the lower enzyme concentrations. This deviation indicates that either physicochemical environment or reaction mechanism might change when going to the highest enzyme concentration considered here. Because this complex colloidal system is influenced by a number of factors, it is also possible that more than one mechanism is involved in the process of the casein micelle destabilization.

We also found that the destabilization of the renneted casein micelles in the mixed batch reactor proceeds slower than in nonmixed batch reactor. It si possible that the lag time was lengthened in the agitated reaction mixture due to changed distribution of the hydrolyzed κ-casein molecules along the micelle surface.

CONCLUSION

Destabilization of casein micelles in reconstituted skim milk was studied experimentally in both nonmixed and mixed conditions. The process of micelle destabilization was described with a three-step kinetic model accounting for hydrolysis of casein in both stable and partially destabilized micelles. This model was used for the estimation of kinetic parameters of κ-casein hydrolysis proceeding on the surface of casein micelles and kinetic parameters of micelle destabilization. The three-step model introduced here can accurately describe the time course of micelle destabilization for the set of process parameters investigated: the relative enzyme concentration 2—4, the substrate concentration 0.1 g cm^{-3}, temperature 32 °C (see Figs. 1 and 2), in the batch reactor with or without stirring. In the range of lower enzyme concentrations (relative enzyme concentration 2, 2.4, 3), where the hydrolysis of κ-casein is relatively slow, we obtained consistent values of the hydrolysis rate constants for all three experiments in both mixed and nonmixed conditions. These kinetic constants differed from those for the highest enzyme concentration (relative enzyme concentration 4), which indicates either physicochemical environment or reaction mechanism could be different at higher enzyme concentrations. We also found that the destabilization of the renneted casein micelles in the mixed batch reactor proceeds slower than in nonmixed batch reactor.

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