Kinetic Parameters of Casein Protein Coagulation

M. ŠEFČÍKOVÁ, J. ŠEFČÍK, and V. BÁLEŠ

Department of Chemical and Biochemical Engineering, Faculty of Chemical Technology, Slovak University of Technology, SK-812 37 Bratislava

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A process of κ-casein hydrolysis and destabilization of casein micelles by means of a probability model was described. The model was used for the estimation of a kinetic parameter of hydrolysis and a critical fraction of hydrolyzed κ-casein molecules on a casein micelle. The experimental kinetic data determined by an analytical method of Bingham were used for the estimation of enzymatic clotting time, initial rate of the destabilized micelle production and the second-order flocculation rate constant. This model provides an acceptable agreement of the kinetic parameter for different enzyme concentrations.

From the surface of casein micelles chain hydrophilic parts of κ-casein, caseinmacropeptide, with an excess of negative charge protrude into a milk serum as flexible hairs. This phenomenon causes mutual repulsion of casein micelles. There is also repulsion by hydration on close contact of individual molecular chains. Casein micelles are fairly voluminous containing much water. The total effect is called steric repulsion. The stability of casein micelles depends on the negative charge density along the micelle surface. Charge milk serum ions are accumulated near the micelle surface and create the electrical double layer. The flexible hairs are essential in providing stability against flocculation of the micelles [1—3].

Proteolytic enzymes split κ-casein into a caseinmacropeptide and a *para*-κ-casein. The caseinmacropeptide is soluble in a milk serum, the *para*-κ-casein remains associated with the rest of micelle. Properties of casein micelles are changed by hydrolysis, their hydration is much reduced, the zeta potential decreases by about 40 % [4]. These changes must be a result of the loss of the protruding chains of κ-casein. They imply that repulsion between micelles is much diminished, micelles now approach each other, flocculate and aggregate.

It has been demonstrated that the enzymatic hydrolysis follows the Michaelis—Menten kinetics [5], or the first-order kinetics [6]. The rate of enzymatic hydrolysis reaction is usually determined by measuring the release of soluble macropeptide or the formation of *para*-κ-casein resulting from the enzyme addition.

When proteolytic enzymes are added to milk there is no apparent action for some time, coagulation of milk only occurs after a period during which no aggregation is observed. The clotting time is the time after the addition of enzyme until flocks or precipitate can be seen in the film of milk. It is a significant parameter of coagulation. *Green* and *Morant* [7] studied casein micelle aggregation by electron microscopy, viscometry, and visual observation of coagulation. They found out that the aggregation did not start until about 60 % of clotting time elapsed, when the enzymatic action was almost complete. At 60—100 % of clotting time the average size of the micelles linearly increased. The occurrence of a lag period in the clotting milk has given rise to much speculations about the existence of primary stage, enzymatic, and secondary stage, flocculation, in the clotting process.

To express the rate at which dispersed particles are converted to a gel, *Payens et al.* [8, 9] presented a model in which the primary and secondary phases were included. These authors assumed that colloid particles became capable of aggregating, in an exact proportion to the amount of the hydrolyzed protective colloids, by the diffusion-controlled process of the irreversible coagulation of hydrophobic colloids according to von Smoluchowski mechanism. At the coagulation modelling *Payens et al.* [8, 9] used the parameter τ_c called enzymatic clotting time; it is supposed to be the time in which the enzyme would produce such number of flocculable particles, that exists in the steady-state phase of the coagulation process. One possibility to determine this parameter is by extrapolating the linear part of the reaction mixture absorbance increase to the time axis.

From the balance equation for a number of flocculated particles at initial phase of the aggregation process, they derived a dependence between time τ_c, the flocculation rate constant k_s, and the initial rate of production of flocculable particles by enzymatic hydrolysis v

$$\tau \approx \left(k_s \frac{v}{2}\right)^{-\frac{1}{2}} \qquad (1)$$

However, the initial hydrolysis rate of κ-casein mole-

cules cannot be used for the estimation of the flocculation constant k_s of milk casein as casein micelles are substrate of the flocculation. And a micelle does not take part in coagulation until a sufficient number of the available κ-casein molecules on their surface was destroyed (*Green* and *Morant* [7]).

Model

If the number of hydrolyzed κ-casein molecules on the surface of a micelle is such as to cause sufficient decrease of the zeta potential and its hydration, the micelle will be unstable and will have properties similar to a hydrophobic colloid. The number of the hydrolyzed κ-casein molecules on the destabilized micelle will be named the critical number. We shall regard the destabilized micelles as a substrate of the flocculation reaction. Using the below described experimental methods, the time-dependent concentration of destabilized micelles can be obtained.

Three phases of the destabilization process can be distinguished:

1. κ-Casein molecules are gradually hydrolyzed by random collisions of the micelle and enzyme, the number of hydrolyzed κ-casein molecules on a micelle is smaller than the critical number.

2. In the reaction mixture there are micelles, which have a critical or higher number of hydrolyzed κ-casein molecules; they are destabilized, their properties are similar to the properties of hydrophobic colloids. The concept of an irreversible diffusion-controlled aggregation of such colloids can be a useful tool in the description of the enzymatic casein coagulation. In these conditions, flocculation can be described by the von Smoluchowski mechanism and the second-order rate constant can be estimated from eqn (*1*), if the enzymatic clotting time and the initial rate of the destabilized micelles production will be used.

3. Both the hydrolysis and destabilization are ended, aggregation is going on.

The enzyme hydrolysis of κ-casein follows the reaction scheme

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

where S, E are κ-casein and enzyme, respectively and P, G are *para*-κ-casein and glycomacropeptide, respectively.

$$\dot{s} = -k_1 s e \qquad (2)$$

$$\dot{p} = k_1 s e \qquad (3)$$

where s, e, p are concentrations of substrate, enzyme, and product, respectively, and $\dot{s}$, $\dot{p}$ are the rates of the concentration changes.

Provided that the enzyme concentration is constant, this reaction may be described by the first-order rate expression [6].

The balance equation of substrate is as follows

$$s_0 = s + p \qquad (4)$$

We defined the dimensionless concentration of κ-casein and *para*-κ-casein x, z

$$x = \frac{s}{s_0} \qquad z = \frac{p}{s_0} \qquad (5)$$

and parameter A

$$A = k_1 e_0 \qquad (6)$$

After rearrangement one gets the equation

$$\dot{z} = A(1 - z) \qquad (7)$$

The stability of a casein micelle in a milk serum depends on the number of the hydrolyzed κ-casein molecules on the casein micelle. To describe the probability that the micelle containing n κ-casein molecules has just k hydrolyzed κ-casein molecules, we used the binomical probability distribution. The dimensionless concentration of the destabilized micelles at time t, $\theta(t)$, will be the sum of probability $P(n, k)$ within the interval of the number of hydrolyzed κ-casein molecules from k_d to n

$$\theta(t) = \sum_{k=k_d}^{n} P(n,k) = \sum_{k=k_d}^{n} \frac{n!}{k!(n-k)!}(z(t))^k (1 - z(t))^{n-k} \qquad (8)$$

where z is the dimensionless concentration of product, k_d is the critical number of hydrolyzed κ-casein molecules.

The dependence of the concentration of destabilized casein micelles on time can be obtained by numerical integration of eqns (*7*) and (*8*). The parameters A and k_d/n were evaluated by the Rosenbrock optimization method. Numerical integration of eqns (*7*) and (*8*) was performed by the Runge—Kutta—Merson method. We estimated the initial rate of the destabilized micelle production v_d from the experimental dependence of the concentration of the destabilized casein micelles on time as the derivative of the linear part of this dependence. The enzymatic clotting time τ_d was determined from the point of intersection of the tangent to the linear part of the dependence of the concentration of destabilized casein micelles on time and the time axis.

The second-order flocculation rate constant can be estimated from the relationship

$$k_s \approx \frac{2}{\tau_d^2 v_d} \qquad (9)$$

MATERIALS AND METHODS

The reconstituted skim milk solutions (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 Chr. Hansen Hannilase powder (Chr. Hansen's Lab. Denmark A/S, Copenhagen, Denmark) was used.

50 cm^3 of reconstituted milk was equilibrated in a mixed batch reactor for 30 min at the reaction temperature 32 °C before any enzymatic reaction was started. The stirrer frequency was 450 min^{-1}. To start the reaction, enzyme was injected into milk.

At predetermined reaction time the reaction was stopped by adding 1 M-NaOH, pH adjusted to 8.2 and cooled to 5 °C. 1 cm^3 of acetate buffer at 5 °C was added to 1 cm^3 of reaction mixture. This solution with pH 5.2 was centrifuged for 15 min at temperature 5 °C. Clotted casein micelles were separated, dissolved in 15 cm^3 of 1 M-NaOH and 25 cm^3 of water was added. Protein was determined by ultraviolet absorption at 290 nm [10, 11]. The relative concentration of clotted micelles was determined as the ratio of the absorbance at specific time t to equilibrium, final value of absorbance at $t \to \infty$.

RESULTS

The kinetic features of casein micelle flocculation have not been studied yet in a broad extent. The presented model combined with the analytical method of Bingham for clotting of the micelles with partly hydrolyzed κ-casein molecules enables a view into the process of destabilization of renneted micelles.

Experimental kinetic data, time dependence of relative concentration of destabilized micelles are shown in Fig. 1 for three enzyme concentrations. These data were used in the optimization procedure with eqns (*7*, *8*). Optimal values of the parameters A and k_d/n are given in Table 1. Solid lines in Fig. 1 represent the relative concentrations calculated using optimal values of the parameters in eqns (*7*, *8*).

To obtain the hydrolysis rate constant k_1 from eqn (*6*), a concentration of the enzyme should be known. We used the relative enzyme concentration. As the unit we chose 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. The hydrolysis parameter k_1 was evaluated from eqn (*6*) for three enzyme concentrations. Calculated values of k_1 are in Table 1 as well as the experimental (visible) clotting time τ_v for the three enzyme concentrations used.

The enzymatic clotting time τ_d and the initial rate of the destabilized micelle production v_d were estimated from the experimental kinetic data by the method described above. The second-order flocculation rate constant k_s was formally estimated by means of eqn (*9*) supposing equality in this relationship.

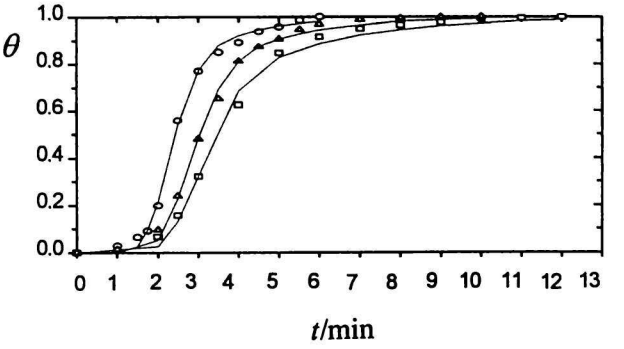

Fig. 1. Dependence of the relative concentration of destabilized casein micelles on time relative concentration of rennet ○ 4, □ 3, △ 2.4.

Table 1. Results of the Evaluation

	Relative enzyme concentration		
	4	3	2.4
A	0.655	0.483	0.366
k_d/n	0.80	0.77	0.70
k_1	0.16	0.16	0.15
τ_v	12.7	15.4	19.5
τ_d	1.8	2.2	2.3
v_d	8.1	5.0	3.8
k_s	0.076	0.083	0.099

A – hydrolysis parameter (min^{-1}), k_d/n – destabilization parameter, k_1 – hydrolysis rate constant (min^{-1}), τ_v/min is clotting time at which the clot becomes visible, τ_d/min – enzymatic clotting time, v_d/min^{-1} – initial rate of the destabilized micelle production, k_s/min^{-1} – the second-order flocculation rate constant.

Results of the evaluations are listed in Table 1. The developed model provides an acceptable agreement of constants for individual measurements.

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