Modification of Colloidal Stability of Casein Micelles by Enzymatic Hydrolysis*

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Destabilization of casein micelles in reconstituted skim milk due to the enzymatic hydrolysis was studied at 32 °C with solutions containing from 0.090 to 0.110 g cm^{-3} of skim milk powder at pH 6.4. Destabilized micelles were precipitated by the acetate buffer and dissolved in NaOH solutions, where their concentration was determined spectrophotometrically. Within the narrow range of skim milk concentrations around 0.10 g cm^{-3} the kinetics of the micelle destabilization process changed rapidly and qualitatively, indicating changes in the destabilization mechanism. While in the solution with the 0.090 g cm^{-3} skim milk concentration the destabilized micelles were produced shortly after beginning of the hydrolysis, in the more concentrated mixture a substantial lag phase exists, leading to a considerably different time evolution of the destabilized micelle concentration. According to the analysis based on the mathematical model of the destabilization process, these changes can be attributed to the shift of the hydrolysis kinetics from the first order to a two-step mechanism and to the change of character of the spatial enzyme activity from the locally correlated action to the spatially random hydrolysis.

Casein micelles in milk are stable agglomerates formed by an association of casein molecules. The micelle interior contains the more hydrophobic α_{s1}, α_{s2}, and β caseins, while the hydrophilic, negatively charged *C*-terminal parts of the κ-casein molecules, glycomacropeptides, are located in the outer micelle layer, protruding out from the micelle as flexible hairs into the milk serum surrounding micelles. The stability of casein micelles against aggregation is primarily due to steric repulsion caused by the glycomacropeptide hairs of κ-casein.

Casein micelles can become unstable and undergo aggregation if hydrophilic *C*-terminal macropeptides are removed from κ-casein molecules at the micelle surface. A proteolytic enzyme hydrolyzes the κ-casein molecule at the specific bond located at the Phe_{105}-Met_{106} site along the molecule backbone, the soluble glycomacropeptide corresponding to the *C*-terminal end of κ-casein is released from the micelle into the milk serum. The *N*-terminal end of the κ-casein molecule, *para*-κ-casein, is insoluble and remains associated with the micelle.

The process of the κ-casein hydrolysis to *para*-κ-casein and its further coagulation was described by *Payens* [1] with the following scheme

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{SE} \xrightarrow{k_3} \mathrm{P}_1 + \mathrm{G} + \mathrm{E} \qquad (A)$$

$$i\mathrm{P}_1 \xrightarrow{k_s} \mathrm{P}_i \qquad (i = 2, 3 \ldots) \qquad (B)$$

where E is the enzyme, S is the κ-casein (hydrolysis substrate), G is the released glycomacropeptide, and P_1 is the *para*-κ-casein (hydrolysis product). Eqns (*A*) and (*B*) formally describe two subsequent steps of the overall process, the chemical reaction of hydrolysis and the physical process of coagulation. The kinetic scheme in eqn (*A*) is generally accepted for the hydrolysis step with various values of parameters, interpreted as either the Michaelis—Menten mechanism [2, 3] or the first-order kinetics [4]. The scheme in eqn (*B*) models the coagulation process, described as a set of bimolecular aggregation processes according to the classical Smoluchowski mechanism, where k_s is the rate constant of coagulation [1]. Although it is

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tempting to implement the Smoluchowski equation for a quantitative description of κ-casein coagulation, first we have to consider what is an appropriate definition of the "substrate" for the aggregation process. The hydrolysis step described by eqn (*A*) is the reaction of enzyme with κ-casein molecule yielding the product – *para*-κ-casein molecule. However, this product is still tied to the rest of the casein micelle by strong protein—protein interactions, so that the substrate for aggregation should be the casein micelle. The ability of micelle to aggregate could be characterized, for example, by an extent of the κ-casein hydrolysis on the micelle surface.

Dalgleish, Brinkhuis, and *Payens* [5] used light scattering to measure the molar mass increase with time after addition of the rennet to very dilute casein solutions containing less than 0.1 % of caseins contained in milk. After the initial lag stage and a short intermediate stage, the molar mass became linearly proportional to time. It was shown that more than 85 % of the κ-casein had been split before any increase in molar mass became apparent. The linear stage of the curve for the molar mass increase occurs when virtually all of the κ-casein has been split by the enzyme. Similar results were presented by *Green et al.* [6] using electron microscopy to determine the degree of aggregation of renneted milk.

Many studies since the works of Payens and coworkers on hydrolysis and coagulation of casein micelles have yielded a wealth of information about the structure and characteristics of micelles [2, 4, 7—9] and hydrolysis in various specific conditions [3, 10—12]. Furthermore, more precise experimental techniques have been introduced [2, 10, 13] and a profound progress was reached in understanding, interpretation, and mathematical modelling of coagulation.

But reliable turbidimetric [3, 12] and light scattering [13] experiments have been conducted in highly diluted milk solutions. Furthermore, this approach provides only information about those micelles that are already a part of the coagulate, while the destabilized micelles which are able to aggregate but have not yet done so are not detected. Viscosimetric methods cover a wide interval of micelle concentrations but the correlation of viscosity and coagulate concentration is more ambiguous than the information provided by the preceding methods.

In addition, milk samples used in various experiments have got different characteristics due to their natural origin and these differences are not readily quantifiable. This can be the reason why even for the hydrolysis stage, which is much better accessible experimentally than the coagulation one, there have been different kinetic models used and the rate coefficients in them are widely scattered [4].

In this work we attempt to contribute to the solution of this problem by using a different, although time-consuming and perhaps less elegant, but nevertheless informative technique based on the observations of *Bingham* [14], who studied the action of the rennet on κ-casein. Her experiments showed that the indication of rennin cleavage of κ-casein is its precipitability in acetate buffer at pH 5.2 and 5 °C. Precipitated proteins were then dissolved in NaOH solution and their concentration was determined spectrophotometrically. We used this technique to determine the concentration of destabilized micelles, *i.e.* the intermediate in the hydrolysis-coagulation process in skim milk solutions.

THEORETICAL

Despite the essential clarification of basic ideas and new insights gained from experiments, the model of hydrolysis and coagulation of casein micelles, formally described by eqns (*A*) and (*B*), still represents the basis for understanding of this process. As mentioned above, the destabilization of the casein micelle is brought about by the limited hydrolysis of its κ-casein component. Since the micelle surface contains thousands of κ-casein molecules, the relation between the overall extent of κ-casein splitting and the number of unstable micelles is not straightforward. Obviously, the concentration of the destabilized micelles must increase monotonously with the degree of κ-casein hydrolysis.

The character of this dependence has not been interpreted unambiguously. The assumption about the systematic activity on one micelle until the complete hydrolysis of the surface κ-casein is still used implicitly or explicitly by many researchers, implying that the destabilized micelle concentration is linearly dependent on the overall degree of hydrolysis. Another assumption, suggested by *Dalgleish* [2], is that of the randomly localized enzyme activity on micelle population with the additional premise about the binomial distribution of micelles according to their ability to coagulate, related to reaching the critical degree of casein hydrolysis in a micelle. This ambiguity is partly due to the fact that there are not generally used direct methods to quantify the intermediate state of the overall hydrolysis-coagulation process, represented by the destabilized micelles in solutions.

To give another insight into this process a model was developed for the hydrolysis of casein micelles resulting in their destabilization, the extent of which can be measured by a procedure based on *Bingham* [14] information. The model is based on the following phenomena and assumptions**.** The first stage of micelle destabilization is the proteolytic hydrolysis of κ-casein to *para*-κ-casein and glycomacropeptide. The hydrophobic *para*-κ-casein remains as a part of the casein micelle, while the soluble glycomacropeptide is liberated to the milk serum. Gradual removing of chains at the micellar surface diminishes the stability of the micelle. A micelle is destabilized only after

a critical proportion of its κ-casein molecules is hydrolyzed.

From this point of view a partly hydrolyzed but yet-not-destabilized micelle can be considered as a complex of substrate and product molecules in a dynamic state, where the number of product molecules increases and the number of substrate molecules decreases. The total amount of molecules remains constant. With regard to the complexity of the process mechanism we suggested [15] to aproximate the micelles destabilization by an effective association between the substrate and the product, which are assumed to form virtually a simple 1:1 complex. Until a critical portion of the surface κ-caseins is hydrolyzed, the complex represents a unit remaining as an individual particle in the milk serum. When a critical extent of hydrolysis of surface κ-caseins is achieved, the micelle is destabilized and thus it can aggregate. The generation of the destabilized micelles is modelled by releasing the product from the complex. The experimentally measured amount of casein in destabilized micelles can thus be identified with the product.

Two subsequent processes of the casein hydrolysis and gradual micelle destabilization were thus modelled by a three-step sequence of hydrolysis, complex formation and complex disintegration by continuing hydrolysis. The hydrolysis process of casein molecules on micellar surface is the same chemical action regardless of whether it is disintegrated as the first or the last molecule or whether the object is the substrate or substrate—product complex. It is appropriate then to use the same rate constants for either case. We used a two-step irreversible mechanism of enzyme-catalyzed hydrolysis according to

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

where the hydrolysis substrate S is κ-casein at the micelle surface, the hydrolysis product P is *para*-κ-casein, the glycomacropeptide G is the side-product of hydrolysis and E is enzyme.

The second step represents the formation of complex C between the substrate and the product

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

Unchanged κ-casein molecules in this complex are still hydrolyzed by the same mechanism as in the first step regardless of the degree of micelle destabilization

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

Rate equations for the three steps of the reaction scheme according to expressions (C—E) were used together with the mass balance equations for the substrate and the enzyme.

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

The initial concentrations of these species are

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

We used the dimensionless concentrations x_1, x_2, x_3, x_4 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 following kinetic parameters were introduced

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

After substituting eqns (*2*) and (*3*) and the mass balance equations for the substrate and the enzyme into the standard mass action kinetic equations for the three steps of the reaction scheme (C, D, E), we get the following set of equations

$$\frac{\mathrm{d}x_1}{\mathrm{d}t} = -Ax_1x_3 - Cx_1x_2 \qquad (4)$$

$$\frac{\mathrm{d}x_4}{\mathrm{d}t} = Cx_1x_2 - Ax_3x_4 \qquad (5)$$

$$\varepsilon \frac{\mathrm{d}x_3}{\mathrm{d}t} = -Ax_1x_3 + B(1 - x_3) - Ax_3x_4 \qquad (6)$$

$$\varepsilon \frac{\mathrm{d}x_2}{\mathrm{d}t} = -Cx_1x_2\varepsilon + B(1 - x_1 - x_2 - 2x_4) \qquad (7)$$

Supposing $e_0/s_0 = \varepsilon \ll 1$, which is suitable for our system, we can neglect terms multiplied by ε in eqns (*6*) and (*7*) and two algebraic equations are obtained

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

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

Then we get the resulting model containing two differential equations (*4, 5*) and two algebraic equations (*8, 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 the resulting system of eqns (*4, 5, 8, 9*), parameter fitting, and statistical analysis were performed using standard numerical procedures [16] and the Statistical Toolbox in Matlab version 6 (MathWorks).

EXPERIMENTAL

Milk solutions were prepared from bovine commercial nonfat skim milk powder Laktino (Promil-PML Nový Bydžov, Czech Republic) reconstituted to powder concentration 0.090, 0.095, 0.100, 0.105, 0.110 g cm^{-3} in 0.01 M-$\mathrm{CaCl_2}$, pH of solution was 6.4. Milk powder Laktino contains in mass %: fat – 0.5, protein – 33, lactose – 52, water – 5, and mineral substances.

A commercial rennet Chr. Hansen Hannilase powder (Chr. Hansen's Lab. Denmark A/S Copenhagen, Denmark) diluted in phosphate buffer of pH 6.2 was used. The phosphate buffer was prepared by mixing 100.45 g of $Na_2HPO_4 \cdot 10H_2O$ and 12.56 g of $C_6H_8O_7 \cdot H_2O$ with distilled water to a volume of 2000 cm^3. 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 50 cm^3 of milk solution with nonfat skim milk concentration of 0.1 g cm^{-3} at temperature 35 °C to the state at which the clot of casein micelles became visible in 40 min.

All experiments were made with 50 cm^3 of reconstituted milk at the reaction temperature of 32 °C in a batch reactor. The substrate solutions were equilibrated in the reactor for 30 min and then the corresponding amount of enzyme was added.

At predetermined reaction times the enzymatic reaction was stopped by adding 1 M-NaOH. Then, pH was adjusted to 8.2 by a solution of NaOH and the reaction mixture was cooled to 5 °C. To clot the destabilized micelles the acetate buffer was used, prepared by mixing of 200 cm^3 of 1 M-NaOH and 490 cm^3 of 1 M-CH_3COOH with deionized water to a volume of 1000 cm^3. One cm^3 of acetate buffer was added to 1 cm^3 of the reaction mixture at 5 °C [17]. This solution with pH 5.2 was centrifuged for 15 min at temperature of 5 °C at 2000 g. Clotted casein micelles were separated, dissolved in 15 cm^3 of 1 M-NaOH and 25 cm^3 of water was added. The protein content was determined by ultraviolet absorption at $\lambda = 290$ nm [14, 17, 18]. 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. Eight repetitions of every experiment were done.

The initial substrate concentration s_0 corresponds to the κ-casein concentration at the surface of micelles in the reconstituted milk solution. The numerical value of the initial substrate concentration, can be assessed only 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 skim milk concentration of 0.1 g cm^{-3}.

RESULTS AND DISCUSSION

The rate of κ-casein hydrolysis depends on process conditions, such as temperature, enzyme and substrate concentrations, hydrodynamic conditions in the reactor, pH and the ionic strength of the reaction mixture. We studied the effects of temperature and enzyme concentration previously [15, 19]. To obtain the standardized reaction substrate we used reconstituted solutions of the identical skim milk powder from the same production batch for all experiments reported here.

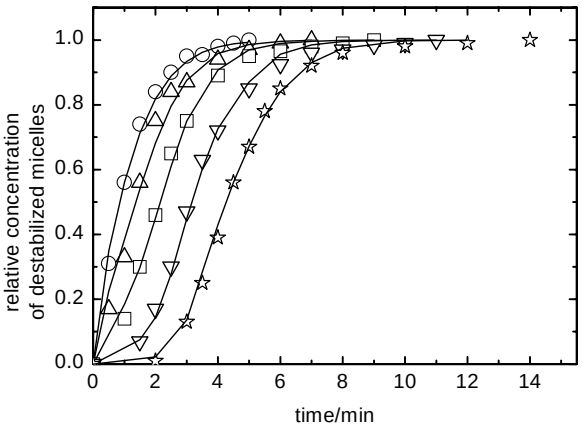

Fig. 1. Time dependence of the measured relative concentration of destabilized casein micelles x_2 at the pH of reaction mixture of 6.4 and the relative enzyme concentration $e_0 = 3.7$ (corresponding to enzymatic activity characterized by the coagulation time of the first visible clot 12 min). Relative concentration of skim milk: ○ 0.90, △ 0.95, □ 1.00, ▽ 1.05, ⋆ 1.10.

In this paper we present results of the measurements on destabilization of casein micelles by enzymatic hydrolysis at temperature 32 °C, pH 6.4, and the constant relative enzyme concentration $e_0 = 3.7$ for five concentrations of dried skim milk solutions. Fig. 1 shows that by the change of the skim milk concentration within ± 10 %, the kinetics of micelle destabilization was qualitatively changed in a manner that could not merely be attributed to the change in the kinetics of the hydrolysis reaction due to varying micelle number concentration. Not only the mutual position of curves representing the time dependence of the relative concentration of destabilized micelles, but also their shape were significantly changed. The initial stage of formation of the destabilized micelles had a different course at various substrate concentrations. At the skim milk concentration of 11 % this dependence had the course typical for the hydrolysis-destabilization process, with the characteristic sigmoidal shape and a well developed lag phase, during which a negligible quantity of destabilized micelles was formed [2, 3, 9, 14]. On the contrary, the formation of destabilized micelles began from the beginning of the hydrolysis process at the skim milk concentration of 9 %. This observation obviously indicates substantial changes in the mechanism of the micelle destabilization process itself.

Without a detailed knowledge of the causes for the change in the process mechanism on the level of molecular interactions in the reaction mixture (with regard to steric factors and electrical phenomena in colloidal media), the analysis of the kinetic model for hydrolysis and destabilization of micelles leads us to propose that in the interval of substrate concentrations consid-

Table 1. Estimated Kinetic Parameters

s_0	A	B	C
	$\min^{-1}$	$\min^{-1}$	$\min^{-1}$
0.90	1.20 (1.0—1.3)	>2	0
0.95	1.00 (0.89—1.15)	>2	8.70 (3.8—13.0)
1.00	0.80 (0.69—0.91)	2.30 (1.6—10)	12.0 (7.3—17.0)
1.05	0.75 (0.67—0.84)	0.61 (0.5—0.72)	21.0 (12.0—30.0)
1.10	1.20 (0.96—1.5)	0.24 (0.22—0.26)	50.0 (19.0—82.0)

The values in parentheses express the 95 % confidence intervals of estimated parameters.

ered, the pattern of spatial enzymatic activity changed from correlated activity localized at a specific micelle to spatially random hydrolysis action.

To quantify these influences we used the set of model equations (*4, 5, 8, 9*) and estimated optimal values of parameters A, B, C by fitting experimentally determined values $x_2 = f(t)$ by using the nonlinear method of least squares. The estimated parameter values are listed in Table 1. Corresponding theoretical curves calculated with optimal values of parameters are shown as lines in Fig. 1.

The results of the parameter evaluation listed in Table 1 provide some basic information about the course of the process. Parameter A, proportional to the rate constant k_1, which is a measure of the enzyme activity, is not changed with varying substrate concentrations and stays essentially constant (*i.e.* with overlapping confidence intervals) in all experiments.

Parameter B, which is proportional to the rate constant k_2, increases strongly with decreasing substrate concentration. The rise of the rate constant k_2 corresponds to the transition of the reaction mechanism according to eqn (C) from the two-stage mechanism to the first-order kinetics (when $B > 2$). For the two lowest substrate concentrations the value of parameter B cannot be determined reliably, since the observed kinetics is not significantly distinguishable from the first-order kinetics.

The influence of the substrate concentration on the mechanism of the hydrolysis process could explain apparent discrepancies between the results reported by various authors studying this process, for example cited in [4]. Some of them presented the enzymatic hydrolysis unambiguously as the first-order reaction, while others described the reaction process by the Michaelis—Menten mechanism with a good agreement between the model and experimental data.

Parameter C, proportional to the rate constant k_3 of eqn (D) which is a measure of the length of the lag phase of the micelle destabilization and subsequent aggregation processes and quantifies the manner of distribution of hydrolyzed molecules of κ-casein on the micelles, changes significantly within the interval of skim milk concentrations investigated.

The low value (not significantly different from zero) of this parameter at the lowest substrate concentration represents a low measure of blocking of the molecular product of hydrolysis by substrate, as is given by eqn (D). It means that already at the beginning of the process the hydrolyzed κ-casein molecules are placed on a relatively small number of micelles with a high degree of local hydrolysis and thus these micelles become unstable. The increase of the value of parameter C with increasing concentration of skim milk in the solution corresponds to the increased position randomness of the enzyme action with more even distribution of hydrolyzed molecules among the population of individual micelles.

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