UDC 637.5.02 DOI: 10.21323/2618–9771–2019–2–3–4–8

The Original Scientific Article

STUDY OF THE ENZYMATIC STAGE OF MILK GELATION: CHANGES IN VISCOSITY AND MICROSTRUCTURE

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KEY WORDS:
milk, rennet gelation, enzymatic stage, casein micelles, viscosity, microstructure

ABSTRACT

The article presents the results of experimental joint studies of changes in the viscosity and microstructure of milk at the enzymatic stage of gelation. Based on the statistical processing of the array of research results, it was determined that the viscosity change at this stage is not monotonic, as it is usually stated, but two-stage in the middle part and S-shaped, preceding the gel point, at its end. It was found that the S-shaped change in viscosity at the end of the enzymatic stage of milk coagulation coincides with changes in the microstructure of casein micelles and reflects the existence of a cooperative conformational phase transition in casein molecules of micelle clusters. A description of the possible mechanism of this phase transition is proposed. It was noted that the moment of the S-shaped change in the milk viscosity at the enzymatic gelation stage and the corresponding cooperative phase transition in casein micelles are a physical reflection of the gel point. The research results provide a better understanding of the mechanism of enzymatic coagulation of milk in a cheesemaking tank.

1. Introduction

Milk gel formation is the key to the technological process of cheese production, one of the main dairy products. Therefore, a large number of scientists' investigations from many countries are devoted to the study of this process, and the development of gelation models and the identification of effective management ways of the gelation in milk.

By now, the rennet coagulation process of milk, according to the generally accepted opinion, can be divided into two main phases: primary (enzymatic), including κ-casein hydrolysis to form para-κ-casein micelles and partial aggregation of destabilized micelles; secondary (non-enzymatic) phase of aggregation, including complete aggregation of micelles into a single spatial structure of the gel and its densifying. At the enzymatic stage, chymosin causes the hydrolysis of the C-terminal part of κ-casein, destabilizing micelles. This hydrolysis produces para-κ-casein (N-terminal part of κ-casein), which remains attached to native micelles, and glycomacropeptide (C-terminal part), which is mainly released in whey. Since the glycomacropeptide has a sufficiently high charge, the total electrostatic potential of casein micelles decreases, weakening the electrostatic repulsion between the micelles. The hydrolysis of κ-casein also eliminates another factor stabilizing the micelles: steric repulsion due to the hydration layer. When a sufficient decrease in repulsive forces (electrostatic and steric) is achieved, the nearest or adjacent micelles begin to aggregate due to hydrophobic interactions. These conditions are achieved at the end of the first and beginning of the second stage of coagulation [1] with a degree of hydrolysis of κ-casein of about 80% and at a pH of 6.6 [2]. These two stages partially overlap in time, but are generally well defined.

The mechanism of the enzymatic stage is well described in molecular terms in [3,4,5,6]. The influence of various environmental factors on it was quantified. Works [7,8] note that the milk viscosity at the beginning of the enzymatic stage of milk gelation decreases slightly and by the end increases again. The experimentally confirmed decrease in the diameter of casein micelles, which occurs due to the enzymatic removal from the surface of micelles of the glycomacropeptide portions of κ-casein, explains this phenomenon.

The initial aggregation of rennet-hydrolyzed casein micelles at the enzymatic stage is confirmed by a series of microphotographs obtained by different authors [9,10]. It is also noted that at the end of the enzymatic stage in casein molecules, cooperative conformational phase transitions that dramatically change the properties of casein micelles occur [10,11,12,13].

Despite the fact that the enzymatic stage of milk coagulation is described in sufficient detail, little attention is paid to studies of changes in the rheological milk properties at this stage. This is probably due to the fact that the enzymatic stage is hidden, there are no visible changes for technological needs and its production control is not required. In addition, changes in the rheological milk properties at the enzymatic stage are very insignificant and are subject to strong influence of external factors. Therefore, the vast majority of studies assume that there are no such changes or they are insignificant. The measurement of viscosity at this stage of gelation is also complicated by the fact that most existing viscometers are unsuitable for these studies. One of the reasons for the impossibility of using mechanical viscometers is the principle of their action — mechanical vibrations that destroy the resulting structure and distort the results obtained. Viscometers with non-destructive measurement methods (optical, ultrasonic, etc.) have low sensitivity in the required measurement range and increased dependence on external influencing factors. However, consideration of the influencing factors and the statistical processing of multiple measurements to a certain extent can compensate these limitations.

Despite the fact that the biochemical processes occurring at the enzymatic stage of milk coagulation have been studied quite deeply and well described, in our opinion, little attention is paid to the change in the rheological milk properties and the changes in the structure of casein micelles.

The purpose of this work is to establish statistically significant changes in the milk viscosity at the enzymatic stage of coagulation and to determine the relationship of these changes with the state and interaction of casein micelles.

2. Materials and methods

Viscometric studies were carried out in the experimental and production workshop of the All-Russian Scientific Research Institute of Butter- and Cheesemaking at the Federal Scientific Center of Food Systems of the Russian Academy of Sciences:

- semi-hard cheese «Rossijskiy» — mass fraction of fat in dry matter 50%, mass fraction of moisture 43%;

FOR CITATION: **Smykov I.T.** Study of the enzymatic stage of milk gelation: changes in viscosity and microstructure. *Food systems.* 2019; 2(3): 4–8. DOI: 10.21323/2618–9771–2019–2–3–4–8

- semi-hard cheese «Kostromskoy» — mass fraction of fat in dry matter 45%, mass fraction of moisture 44%;
- hard cheese «Altaiskiy» — mass fraction of fat in dry matter 50%, mass fraction of moisture 42%,

Cheese production was carried out according to the standard production processes, including heating, normalization at 40 °C and milk pasteurization in a tank at a temperature of 68 °C for 10 minutes, cooling milk, introducing liquid bacterial starter culture, calcium chloride and enzyme preparation, mixing and coagulating milk at a temperature 34±1 °C, cutting and further operations on the cheese formation.

Studies used cow's milk of one supplier-manufacturer — «AgriVolga» LLC, village Burmasovo, district Uglich, Yaroslavl region, Russia.

In the cheeses production, liquid starter cultures based on polyspecies bacterial concentrates «Bioantibut» and «BK-Uglich-5A», manufactured by «Experimental Biofactory», Uglich, Russia, were used.

The studies used «Rennet Enzyme 90, Extra» (chymosin — 90%, beef pepsin — 10%), MSA — 100000. «Plant of endocrine enzymes», Moscow, Zelenograd, Russia.

Measurement of changes in the milk viscosity at the enzymatic coagulation stage was carried out as follows: after pasteurization and introducing the bacterial starter culture and calcium chloride, one liter of the milk mixture was taken from the tank and placed into a capillary viscometer with a volume of one liter and a capillary diameter of 2.3 mm, produced by the «Experimental Machine-Building Plant», Uglich. A capillary viscometer was used only to determine the moment of completion of the enzymatic gelation stage (gel point) by stopping the milk flow through the capillary. Studies of changes in the milk viscosity at the enzymatic stage of coagulation in a capillary viscometer were carried out by introducing an increased dose of rennet, so that the enzymatic stage duration was 2–3 minutes. The capillary viscometer was additionally equipped with an automated system for in-line monitoring of the milk viscosity by changing its thermal conductivity using the hot wire method [14]. As a heated sensor by this method, a TSP-100 platinum resistance thermoconverter with two identical sensitive elements with a resistance of 100 Ω in one housing was used. One of its sensitive elements was used for its intended purpose for measuring temperature, and the other as a heater, powered by voltage from a stabilized standard power supply. The temperature control sensor had similar characteristics, but consisted of one sensor element. The heated and control sensors were connected to a universal microprocessor two-channel measuring instrument that calculates the temperature difference between them, which correlates with the milk viscosity. Control of changes in viscosity and temperature in a capillary viscometer was carried out while recording the research results with an interval of 1 second. In total, the measurements results of the milk gel viscosity at the enzymatic stage of coagulation on 43 developments of marketable cheese were analyzed.

Electron microscopic studies of changes in the microstructure of the protein phase of milk at the enzymatic gelation stage were carried out using an EM-410 transmission electron microscope (Philips, the Netherlands).

Samples for electron microscopy were prepared by direct microscopy with contrasting platinum. For this, a film of parlodium (nitrocellulose) with a thickness of about 40 nm was preliminarily applied to a copper mesh (600 mesh, Electron Microscopy Sciences). Milk samples taken from a capillary viscometer at the desired time points of the enzymatic gelation stage were diluted with distilled water with a ratio of 1:100, fixed with glutaraldehyde, and immediately after that (~10 s), they were immobilized onto palladium-coated copper grids. The nets were dried, then washed twice with distilled water and finally dried. The grids with the sample were placed in a vacuum chamber, which was then evacuated to 10^{-6} torr, and a contrasting layer of platinum (Pt) with a thickness of 3–5 nm was applied to the surface of the etched preparation. For this, the method of thermal vacuum spraying of platinum at an angle of 24° to the plane of the sample was used. A quartz sensor monitored the thickness of the applied material. Electron microscopy studies were carried out at an electron beam current of microscope of 10 μA and a voltage of 80 kV.

Statistical processing of the research results obtained was carried out in the EXCEL program (Microsoft, Redmond, WA, USA), with a significance level of $P < 0.05$.

3. Results and discussion

When conducting experimental rheological studies of milk coagulation under production conditions, it is difficult to ensure reproducibility and precision of the results. This is related both to the lability of the milk properties and the action of external influencing factors. If changing the milk properties under these conditions is practically impossible, then taking into account the influence of some influencing factors is quite accessible to the experimenter.

The milk viscosity can be represented as the sum of the viscosity of water and the increment of viscosities from the dispersed phase (proteins, fats, carbohydrates) and structural bonds [15]:

$$\eta_{m0} = \eta_h + \Delta\eta_d + \Delta\eta_{st}, \quad (1)$$

where:

η_{m0} — the initial value of viscosity;
η_h — the water viscosity;
$\Delta\eta_d$ — the viscosity increment from the dispersed phase;
$\Delta\eta_{st}$ — the increment of viscosity from structural bonds.

According to the technological documentation, the temperature of the milk in a cheesemaking tank at which it coagulates can fluctuate within two degrees, and the milk viscosity, like any other liquid, strongly depends on the milk properties and temperature. The dependence of the dynamic milk viscosity on temperature can be described by the formula [16]:

$$\eta_m = \eta_{m0} + A \cdot \exp b \cdot T, \quad (2)$$

where:

η_m — the milk viscosity;
A — preexponential coefficient;
b — the decrement of the function;
T — the temperature.

At the enzymatic stage of milk coagulation, its viscosity also changes with time. Then, in general, the change in viscosity is determined by the function:

$$\eta_{m0} = \eta_h + \Delta\eta_d + \Delta\eta_{st} + \Delta\eta_t(t) + A \cdot \exp b \cdot T, \quad (3)$$

where the dependence $\Delta\eta_t(t)$ remains completely unexplored.

The above dependences are of a qualitative nature, a quantitative assessment of the values of the components of these dependencies in production conditions is practically impossible, and model laboratory experiments do not fully reflect the processes that occur in reality.

In our opinion, one of the approaches for evaluating the processes occurring at the enzymatic stage of milk coagulation in a cheesemaking tank is a statistical analysis of viscosity changes. This approach allows us to identify the systematic components of the viscosity change and separate the random components.

During the research, the results of measuring the viscosity of milk gel on 43 samples during the production of marketable cheese were analyzed. The changes in viscosity and temperature in the capillary viscometer were determined in-line using the hot wire method [14,17] with an interval of 1 second during automatic

recording of the research results using a PC with the OWEN Process Manager preinstalled program.

Processing the array of research results was carried out in several stages. As the temperature of the milk in the cheese-making tank and its technological properties were different on different days, but within the technological norm, and the amount of rennet introduced was also different, the duration of the enzymatic coagulation stage was also different. Therefore, at the first stage of processing the data array, they were scaled, i.e. all groups of research results due to compression or extension were reduced to a single duration of 120 seconds. At the same time, the moment the rennet was introduced was taken as the beginning of the process, and its end was determined by the moment the milk flow stopped through the capillary of the viscometer.

During the research, it was found that the temperature of the milk in the viscometer somewhat varied differently on different days, which also significantly affected the research results. To eliminate this influence, at the second stage of processing, in each group of research results, they were adjusted in accordance with dependence (2).

At the final stage of processing the research results, they were grouped again by time intervals with the calculation of the average value in each interval. The calculated average values of the milk viscosity at certain time intervals of the enzymatic coagulation stage were used to display graphically its change in time (Figure 1).

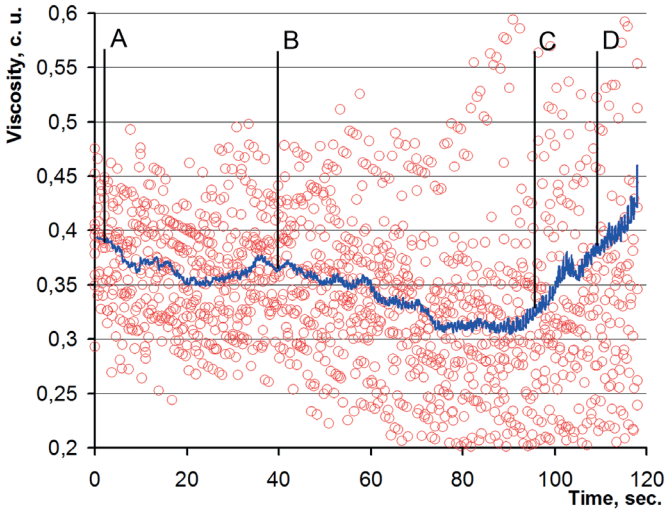

Figure 1. Changes in the milk viscosity at the enzymatic gelation Stage. Multiple measurements

The graph clearly shows that, in general, the milk viscosity at the enzymatic stage of coagulation first decreases and then increases. This fact has long been known [7,8] and this phenomenon is most often explained by the experimentally confirmed decrease in the diameter of casein micelles due to the enzymatic removal of the glycomacropeptide portions of κ-casein from the micelle surface, and its detection in our experiment by means of the used hot wire method confirms the correct choice of measurement method and high sensitivity of the device.

At the same time, the graph obtained by us shows the decrease in viscosity has two distinct regions not described in the literature known to us, primary (AB) and secondary (BC). These sections are separated by some increase in viscosity, i.e. the decrease in viscosity at the enzymatic coagulation stage is not a monotonic process, and cannot be simply explained only by the removal of glycomacropeptide sites from the casein micelles surface. This issue requires additional, multilateral and thorough research and may be the subject of independent study.

In addition, in the final section of the graph obtained by us (Fig. 1), a well pronounced S-shaped bend is observed preceding the gel point, after which the milk gel viscosity increases sharply. The appearance of such a statistically significant change in the milk viscosity at the end of the enzymatic stage of coagulation required additional studies. In this regard, electron microscopy studies of possible changes in the milk microstructure at this stage were carried out.

Samples were taken from a capillary viscometer during the milk coagulation at specific time points (Figure 1). A is the beginning of the process, B is the middle of the stage, C is the moment before the gel point, and D is the moment after the gel point. Preparations for electron microscopy were made from the selected samples, and then they were photographed and the images obtained were analyzed.

Figure 2 shows electron microscopic images of casein micelles in milk at different points in time of the enzymatic gelation stage. These images clearly illustrate the kinetics of changes in the microstructure of the protein milk phase.

At the beginning of the enzymatic coagulation stage, casein micelles are isolated (Figure 2A), and after partial removal of the glycomacropeptide portions of κ-casein molecules, they have already been forming separate di-, tri- and tetramers (Figure 2B), in which the micelles are still weakly connected to each other hydrophobic and van der Waals interactions. It should be noted here that, as shown in [18], the property of micelles to form loosely coupled aggregates is also observed in freshly drawn native milk, but further technological processing of milk usually eliminates this property of micelles. Any mixing of milk at this stage leads to the breaking of these weak bonds and, as a consequence, to a decrease in the density of the gel.

Then (Figure 2C) weakly bound clusters are formed from di-, tri- and tetramers and individual micelles. The weak nature of the bonds between casein micelles in the resulting clusters is confirmed by an insignificant change in the viscosity of the sample in this section of the stage. The obtained results of electron microscopic studies of the gelation process are close to the results obtained in [7, 8] by other analytical methods, which also indicate the existence of casein micelle aggregation at the enzymatic stage, as a prelude to coagulation [19].

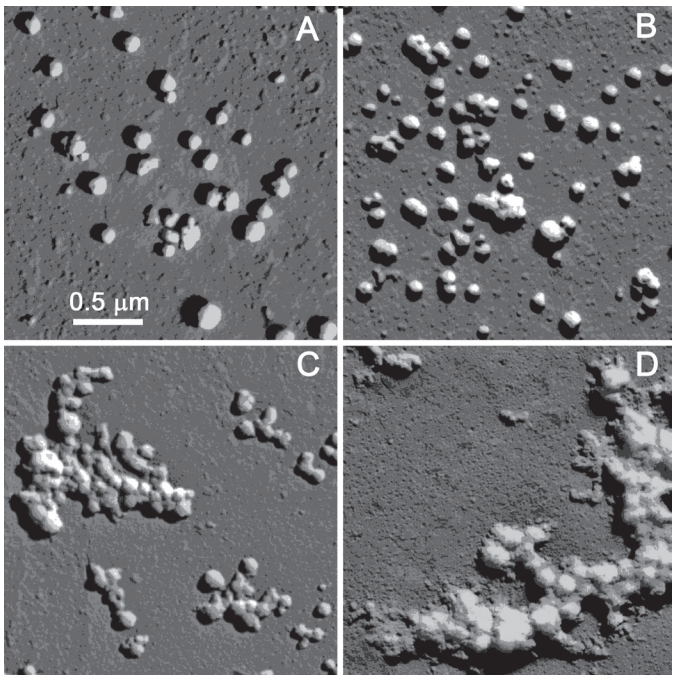

Figure 2. The formation and change of casein micelle clusters at the enzymatic stage

Sharp changes in the structure of casein micelle clusters are observed at the end of the enzymatic stage (Figure 2D). Here the micelles of para-κ-casein itself, i.e., κ-casein lacking glycomacropeptide sites, look strongly deformed, partially destroyed. However, in the future, these clusters become denser due to vibrational and rotational movements of casein micelles inside the cluster and the formation of additional covalent intermicellar bonds due to this. It was precisely such clusters with a compacted structure to be subsequently the starting material for the formation of a three-dimensional spatial structure of milk gel at the secondary stage of gelation.

It can be easily seen that the change in the state of micelle clusters from 2C to 2D morphology coincides with the appearance of a statistically significant peak or, more precisely, an S-shaped change in viscosity on this plot. Immediately after this area, which is logical to take for the physical display of the gel point, an intensive increase in the viscosity of the gel begins, i.e. immediately after the S-shaped site begins the secondary stage of coagulation.

A similar peak can be seen in some graphs given by different authors [20] and obtained by different methods, which allows us to state the non-randomness of this phenomenon in relation to gelation in various dispersed food systems. However, there is a small amount of such works, and this is most likely due to the low sensitivity of the equipment, used in the research, and the small value of the S-shaped change, perceived as a measurement error. According to our data, systemic studies of the S-shaped change in viscosity at the end of the enzymatic coagulation stage were not carried out either in the Russian Federation or abroad.

As can be seen from the above photographs, micelles in clusters undergo significant structural changes. If at the beginning of the S-shaped region, the micelles retained a spherical shape and the bonds between them in the cluster were weak, then after the region there are no spherical micelles in the cluster, they all unfolded, i.e. there was a denaturation of casein molecules in micelles according to the principle of the «all or nothing» transition. This type of phase transition in various proteins has been well-studied [21], and it has been firmly established so far that denaturation is a cooperative transition with a simultaneous and sharp «S-shaped» change in the properties of the protein molecule. The S-shape of the experimental curves obtained by different authors shows that the properties of the molecule vary from those characteristic of a native protein to those characteristic of a denatured protein; and the narrowness of these S-shaped curves indicates the cooperativity of the transition, i.e. that it immediately covers many amino acid residues.

In contrast to the true phase transition, the S-shape of the «all-or-nothing» transition has not a zero but a finite width, since this transition covers not a microscopic, but a macroscopic, small system.

As is known [22,23], casein protein molecules in their secondary structure contain α-helices, β-structures and disordered, statistical tangles. During denaturation in the α-helix, a first-order phase transition does not occur, since the boundary of the helix, unlike the boundary of the β-structure, does not grow with its size, and therefore the barrier to be overcome has a finite small size, and it can turn around in microseconds. When a protein molecule is denatured, its β-structure changes according to the type of phase transition of the first kind, which is characterized by extremely slow initiation — a common property of phase transitions of the first kind, associated with the large free energy of a wide phase boundary of β-structures and the time of this transition can be small fractions of seconds to minutes.

As can be seen from the photographs, in our case, the phase transition is cooperative in nature for all casein micelles in the cluster. However, for various clusters in the system, the cooperativity property is not observed, and the phase transition for the system as a whole occurs over some time (from the gel point to the beginning of syneresis) and is determined by both external conditions and the intrinsic properties of the micelles.

In the process of gelation in the micelle, the transition «dense globule — loose coil» occurs, which is accompanied by an increase in the size of the casein molecule, a change in its physicochemical properties and, as a result, an increase in the penetration ability of water molecules and ions dissolved in it, which contribute to the formation of new covalent bonds inside the micelle and between them. Casein molecule does not have a thermodynamically significant secondary structure in a coil.

After the protein molecules in the casein micelles went into the conformational state of the «loose coil», the access of chemically active components and water molecules in the milk plasma to the previously hidden, internal parts of the protein molecules was facilitated. In addition, there is the possibility of chemical interactions between neighboring sites of neighboring molecules, for example, the formation of disulfide bonds between cysteine residues and calcium bridges. These chemical interactions, in turn, are accompanied by the appearance of local temperature and concentration gradients both on the surface of the micelle cluster and between neighboring clusters. As a result, chemicapillary convection of Marangoni may occur [24]. The probability of the occurrence of concentration-capillary flows in the plasma layer of milk near the surface of micelle clusters is also very high. Flows arising in the microscopic layer entrain the remaining free casein micelles and their clusters, facilitating their interaction and further aggregation into the integral spatial structure of the gel. Microconvection flows in the forming gel act at distances of the order of units of micrometers with velocities of the order of units of micrometers per second and are modeled in the framework of the theory of microconvection.

Thus, in the milk, after the initiation of the gelation process (introduction of the enzyme) and the formation of micelle clusters at the end of the enzymatic stage, cooperative phase transitions occur in them, creating (micro) gradients of temperature, concentration, density, viscosity and surface tension, which cause thermophoresis, thermocapillary, concentration-capillary and other types of microflows, on the surfaces of casein micelle clusters. In turn, microflows cause micelles to move and intensify interactions between clusters and micelles in them, which contributes to their merging with each other and the emergence of new property gradients in clusters, i.e. the emergence of internal feedback between these processes.

In our opinion, at the end of the enzymatic gelation stage in casein molecules forming micelles, and a first-order cooperative phase transition occurs in micelle clusters. This phase transition, firstly, leads to an increase in the size of micelles and, accordingly, their volume fraction, which causes a slight increase in viscosity, and, secondly, there is a sharp change in the physicochemical properties of micelles, which contribute to the further formation of an integral gel structure from them.

4. Conclusion

Using statistical processing of the array of research results of changes in the milk viscosity at the enzymatic gelation stage obtained by repeated production and experimental measurements, it was possible to identify previously unknown systematic components of this process — a two-stage decrease in the milk viscosity in the middle of the process and an S-shaped viscosity change preceding the gel point.

Joint studies of changes in the viscosity and microstructure of milk at the enzymatic gelation stage showed that the S-shaped change in viscosity is caused by a cooperative first-order phase transition in casein micelles.

A description of a possible mechanism for the interaction of casein micelles at this stage of gelation and the processes occurring in micelle clusters during a cooperative phase transition is proposed.

It is proposed to use the moment of the S-shaped change in the milk viscosity at the enzymatic gelation stage and the corresponding cooperative phase transition in casein micelles as a physical reflection of the gel point, after which an intensive increase in the gel viscosity begins and the secondary gelation stage begins.

5. Acknowledgment

The author expresses deep gratitude to Natalya Moshkina, experienced master cheese maker, head of the VNIIMS experimental workshop, for invaluable assistance in conducting experimental studies in the production of cheese.

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Completely prepared the manuscript and is responsible for plagiarism.

The author declare no conflict of interest.

Received 19.06.2019 Accepted in revised 01.09.2019 Accepted for publication 15.09.2019