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Physico-chemical studies on the enzymatic coagulation of milk*

By B. Tarodo de la Fuente** and C. Alais***

Université des Sciences et Techniques du Languedoc
Université de Nancy I

1 Introduction

In a recent publication that appeared in this review[1] Alais outlined our present knowledge of $\varkappa$- and whole casein, of chymosin and other coagulating enzymes. He also described the action of chymosin (rennin) on the casein. In this paper we give a review of what is known on the alterations and the stages of milk and casein following the reaction of the enzyme. Thus we shall consider the actual coagulation or the secondary phase, the paracaseinate gel syneresis and the different methods of studying the two phenomena.

2 Secondary phase of milk coagulation

A) Mechanism

The primary phase namely the proteolytic reaction may be studied by different techniques[2, 3, 4, 5, 6], but unlike the actual coagulation or the secondary phase, it is not visible to the experimenter.

It has been shown that the flocculation time which is measured from the instant of rennet addition to the appearance of the first floccules of casein (which result from high and rapid aggregation of micelles) was inversely proportional to the quantity of glycopeptides released by the chymosin or the propagation of the enzymatic reaction and also to the initial rate of this reaction[7].

The generally accepted hypothesis is that the formation of the paracaseinate gel does not occur unless the stabilising power which the $\varkappa$-casein exerts on the solubility of the other caseins with respect to calcium is destroyed[8]. When this stabilizing power is lost as a result of the enzymatic release of glycopeptides the coagulation can take place if the calcium ion content of the milk is sufficient. However the mechanism of the secondary phase, that is to say the process of micelle aggregation has not been completely elucidated.

The problem of the native micellar structure that influences the reactions of the secondary phase is still not resolved, in spite of a great number of publications, for lack of adequate physical techniques. Electron micrographs of micelles have provided interesting informations chiefly by the investigations of Nitschmann[9], Hostettler and Imhof[10] and Shimin and Hill[11]; yet some observations seem difficult to explain. Among the numerous models proposed for bovine casein micelles certain are audacious intellectual constructions. However two principal models are in competition: 1) a compact structure with $\varkappa$-casein located on the surface of the micelles, covering an inner core of α_s and β-casein[12, 13], and 2) a loose structure which permits the penetration of some enzyme molecules, the three major casein components being distributed uniformely within the micelles[14, 15, 16].

As a result of the work of Green[17] we can reject the theory of Parry and Carroll[18] which centers on soluble $\varkappa$-casein and their micelle model. This theory supposes that $\varkappa$-casein in the milk serum leads to para-$\varkappa$-casein under the action of rennet, which then adheres to the surface of the micelles and forms bridges between them, so causing their aggregation. However, a pre-treatment of milk serum with rennet did not reduce the clotting time of reconstituted milk prepared by addition of such milk serum to a concentrated micelle suspension[17].

The work of Green[17] and Green and Crutchfield[19] has made it rather improbable that the coagulation is mainly caused by calcium bridge formation between the casein micelles. The most favoured hypothesis at present[14, 15, 19, 20] involves hydrophobic interactions between the trimer of $\varkappa$-casein found on the micelle surface and a modification of the electrostatic forces as a result of the action of chymosin or of the fixation of additional calcium which will reduce the negative charge on the micelles. It is believed that as a result of charge diminution the attractive forces between casein particles become greater than the repulsive ones.

The coagulation of native casein depends very strongly on the temperature[21]. The temperature coefficient of this reaction is in fact very high, 1.3 to 1.6 per degree centigrade. In ordinary cow milk gel formation does not occur below 18°[21, 22]. We see here the possibility of the technique described by Berridge[21] to separate the secondary phase of milk coagulation from the primary one, the temperature coefficient of which is much lower, about 2 per ten degrees.

An increase in negative charges on the micelle during cooling and a reduction of attractive forces between the casein particles could explain the absence of coagulation

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** Address: Professeur B. Tarodo de la Fuente, Technologie Alimentaire, Université des Sciences et Techniques du Languedoc, F-34 060 Montpellier

*** Address: Professeur C. Alais, Biochimie Appliquée, Université de Nancy I, F-54 037 Nancy

at low temperature[19]. This charge increase is the result of many factors which will be differently influenced by temperature changes: there are conformational changes in the β-casein, changes in pK of certain groups, charge reductions due to solubilization of parts of the casein and changes involving liberation of calcium ions or fixation of the phosphates[19, 23, 24, 25].

B) Methods of study

The different techniques of observation used to detect milk coagulation and to measure gel evolution may be applied either to the determination of the commencement of the phenomenon (flocculation) or of the changes of the mechanical properties of the curd. They can also be applied to the simultaneous study of casein flocculation and the subsequent paracaseinate gel evolution.

B.1 Flocculation: The easiest way to determine the flocculation time is to use the method of Sommer and Matsen[26]. The technique consists in observing a thin milk film that is formed in a large glass tube inclined at 30° which rotates very slowly about its axis (10 to 12 r/min). The phenomenon to be observed is a coarse fragmentation of this film left on the wall of the rotating tube above the surface of the milk sample. A trained worker can obtain results reproducible within two seconds if care is taken that the flocculation time lies between 6 and 12 minutes. It should be remarked that flocculation precedes the gel formation.

B.2 Viscosimetry: The action of rennin on the casein leads to viscosity changes in the milk during the specific primary phase of coagulation[27, 28, 29, 30, 31]. As shown in Fig. 1 initially a limited drop in viscosity is observed. It occurs simultaneously with the release of non-protein nitrogen by the enzyme (zero order reaction). The angle "a" varies inversely with both the chymosin concentration and the temperature. It could be said that the viscosity minimum corresponds to the completion of the primary reaction. The minimum is reached after a reaction time which is approximately half of the flocculation time. Visible flocculation occurs on the ascending part of the curve where the slope is steep.

In order to measure accurately the small decrease in viscosity at the beginning a reliable apparatus like the one shown in Fig. 2 must be used. It is an Ostwald type automatic instrument furnished with a quartz oscillator electronique stop watch. Studies of the influence of the principal parameters are still going on[32].

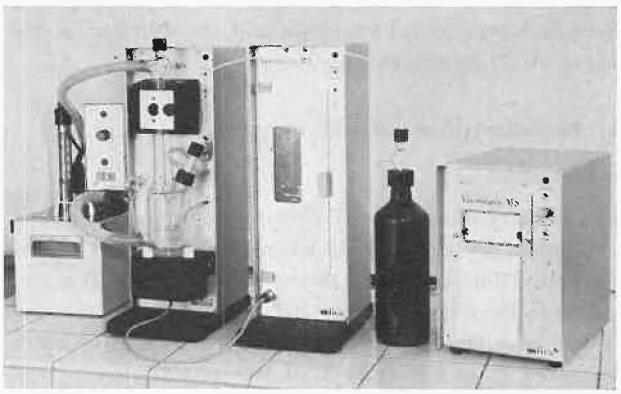

Fig. 2: High precision automatic viscometer VISCOMATIC M.S. FICA. From the left to right: thermostatic bath, actual viscometer, programmed dilutor, printer.

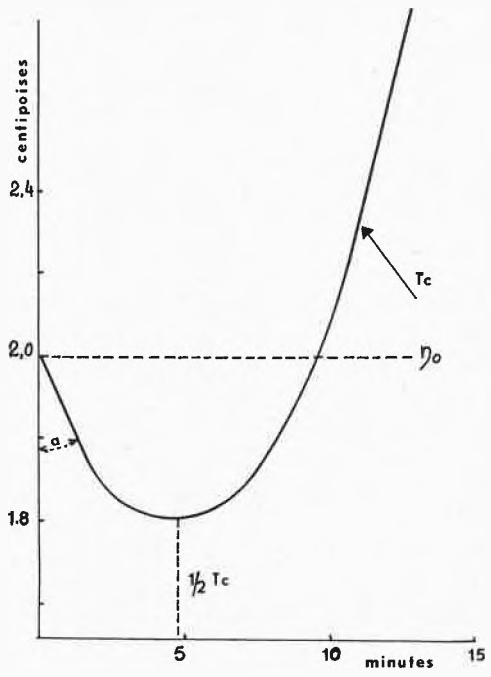

Fig. 1: Viscosity changes in milk at the onset of rennin action[32]. Tc = coagulation time.

B.3 Particle counting: The particle size of the casein changes during the primary phase even before one can detect the least visible signs of flocculation. Several experiments carried out using a particle counter (Coulter Counter) have shown that the increase in size of casein aggregates of diameter around 2 to 3 μm corresponds to the completion of the enzymatic phase[33, 34].

B.4 Electron microscopy: This technique has been largely used to study micellar structure and micelle aggregation under the action of rennin, initially in Switzerland[9, 10, 35], then in other countries[11, 36, 37, 38, 39, 40]. Fig. 3 presents a precocious aspect of the agglomeration of small micelles during rennin action.

Electron microscopy is not appropriate for the determination of the end of the primary phase because the state of the micelles should be fixed before observation which renders continuous observation of this phenomenon very difficult. However it appears that the agglomeration of micelles between themselves which leads to visible flocculation and thus gelification is continuous and accelerated with respect to rennin action[10, 38].

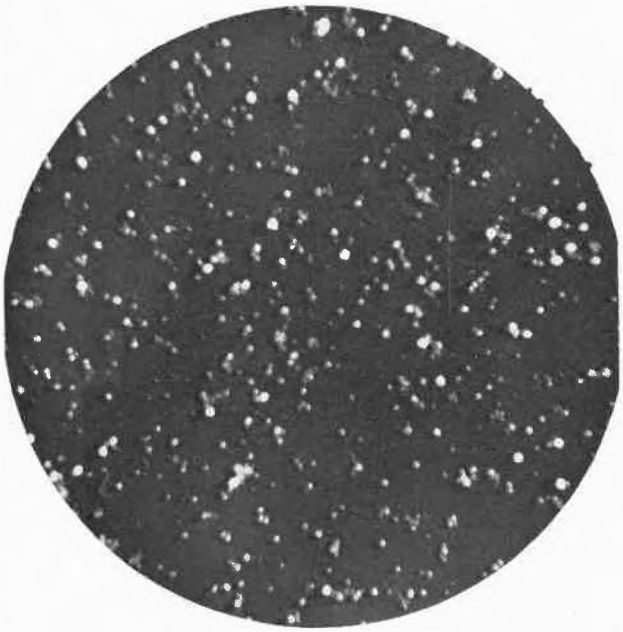

Fig. 3: Shape of casein micelles in cow milk after 14 minutes of rennin action; beginning of aggregation. Magnification × 8000 (Hostettler and Imhof,[10].

B.5 Penetrometry-Torsion measurements: The firmness of the paracaseinate gel or of the curd has always been estimated if not measured in the cheese industry since it determines to a large extent the organoleptic quality of the product. Recent measurements use penetrometry [41,42,43,44,45,46] or torsiometry [47,48,49]. The latter technique, unlike the penetrometry which gives punctuated measurements, is apt to describe continuously a part or the complete evolution of the coagulum.

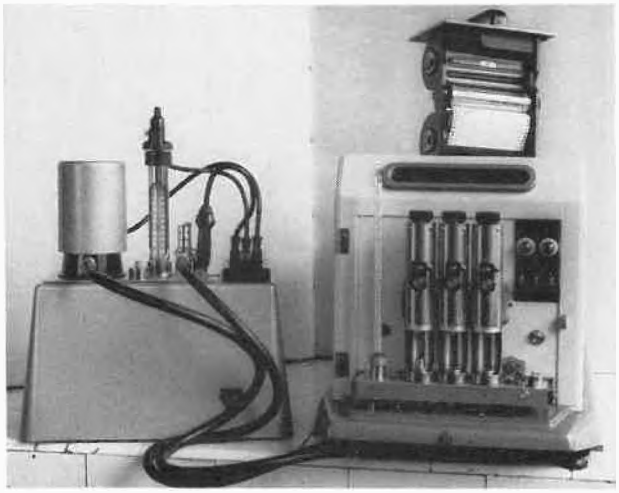

Fig. 4: Thrombelastograph. From left to right: thermostated bath, actual thrombelastograph with the three measuring units. Top of system used in winding the photographic paper.

In this regard, the thrombelastographic method which has originally been developed to study blood coagulation is interesting. It has already been discussed in detail elsewhere[50,51]. Torsiometer and thrombelastograph utilize a system of coaxial cylinders. In the latter, the measuring vat is put into a slow alternating coaxial motion so as not to provoque disruption of the developping gel by shearing. The essential characteristic of the thrombelastographic technique is its ability to allow us to follow the gel formation and the contraction of the gel. However, it must be noted that the flocculation time as measured by the thrombelastograph corresponds to the beginning of the gel formation but not to the appearance of visible flocks. It will therefore necessarily be longer than the time measured with the technique of Sommer and Matsen[26].

Furthermore, the usage of small quantities of milk and rennin makes manipulations very delicate. Careful control of the temperature at which experiments are carried out is required. Fig. 4 and 5 show a thrombelastograph and a scheme of the measuring unit.

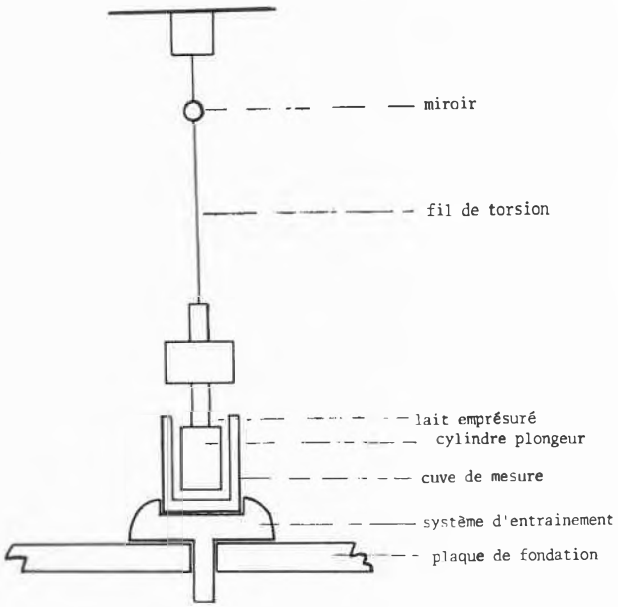

Fig. 5: Scheme of the principle of the measuring unit of a thrombelastograph.

3 Syneresis

A) Mechanism

The mechanism of the syneresis succeeding coagulation has not been clearly elucidated. The agglomeration of casein micelles which causes flocculation continues with the establishment of different types of bonds which will lead to a contraction of the coagulum with expulsion of serum.

Rennin action itself, involving the hydrolysis of $\varkappa$-casein, causes an initial dehydration due to the loss of the most hydrophilic part. Electrokinetic potential measurements confirm this release and the dehydration of the micelles. The protecting sheet of hydration water is thereby reduced thus faciliting the formation of different bonds. These may be due to the appearance of active groups

exposed by molecular rearrangements or by the formation of sulfur bridges[52, 53, 54]. It has been shown that the addition of reagents that are capable of blocking the SH groups, for example salicylamidoacetic acid or N-ethylmaleinimide prevents syneresis. However the introduction of excess cystine reestablishes it[55]. All the Cys residues of the $\varkappa$-casein are found after the enzymatic splitting in the $\varkappa$-paracasein[55]. The syneresis itself does not seem to be accompanied by variations in the hydration of paracasein as shown by calorimetric and sorption studies[56].

It is interesting to remark that factors which are favourable to the production of a firm curd are unfavourable to the syneresis[50]. This is the case with an increase in acidity which causes progressive solubilisation of colloidal phosphocaseinate, hence a reduction in the firmness of the gel produced, but at the same time inducing accelerated syneresis. The increase in total solids and the calcium content of milk produces a reverse effect; it favours the coagulum firmness and slows down syneresis. It seems that the release of active groups following solubilisation of calcium has a relatively great importance. Everything occurs as if the calcium had two actions: on one side it favours the formation of large aggregates and makes the gel very firm, on the other side it occupies active sites which after coagulation could allow the formation of bonds that are favourable to syneresis. The increase of the amount of rennin used is equally favourable to curd-whey separation.

It is difficult to explain the effect of an increase in temperature which favours whey expulsion, yet results in very wet curds. We could in fact expect rapid micelle associations producing very firm gels since the increase in temperature favours such arrangements[13]. It is probable that the bonds which cause curd syneresis are established more rapidly than those which confirm a given firmness to the curd.

These are not the only factors that are involved in the syneresis. It could be said that the various treatments which the milk is subjected to during cheese production play a part and prepare the ground for physical or mechanical processes involved in the technology, such as slicing, stirring, heating, washing and pressing. Several studies have been published on the relative volume of the curd after slicing[57, 58], stirring and washing[59].

B) Methods of study

The large number of parameters involved and the need to work under conditions normally prevailing in cheese industry renders an objective measurement of the syneresis and the whey expulsion which accompanies it very delicate.

B.1 Pitching balance: Scott Blair and Coppen (1940) have developped a balance for measuring the draining of stirred cheddar cheese curd. The principle is simple. Grains of the curd are filled into a cylindre and after draining off the whey for 30 seconds weight and height of the sample are determined. The ratio weight/height decreases with the progress of the cureing of the curd to an value optimum for the cheese making process[60].

B.2 Photometry: Beeby (1959) and Pulay *et al.* (1971) used as a tracer skimmed milk to which formol is added to prevent coagulation or a glycine solution. The tracer is added on top of the curd and a graph is drawn to show the extent of dilution of opaque tracer by the milk serum as a function of time[61, 62].

B.3 Thrombelastography: Tarodo de la Fuente *et al.* (1969) utilised the thrombelastographic technique which has the advantage of giving a continuous recording of the phenomenon[50]. However this direct recording appears incomplete. Until the syneresis expulses increasing quantities of whey, the cylinder which allows us to register the evolution of the gel is gradually driven by the motion of the bowl surrounding it. When syneresis starts and enough whey is exuded the cylinder is no longer driven by the motion and rectilinear plots appear on the photographic paper (Fig. 6) even though the curd syneresis is not yet complete.

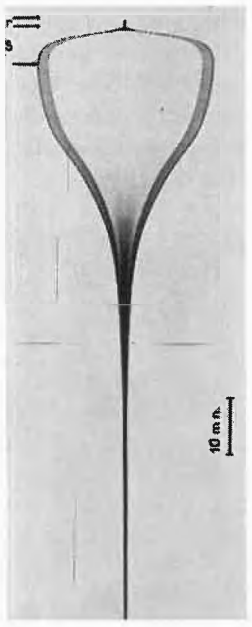

Fig. 6: Thrombelastogram showing a contraction profile at a *p*H of 5.6. *r* measures flocculation time, *S* is the time required for the profile to reach its maximum separation.

B.4 Volumetric estimation: We can also measure the quantity of whey exuded as a function of time in a simple way. The milk is renneted in a thermostated copper tube with an outlet at its base. The tube is provided with a screen which allows the curd to be lifted without breaking it, so that the whey can flow freely into a fraction collector. Unlike with the methods described in B.2 and B.3 the curd no longer stays in the serum and so the syneresis is favoured[59].

4 Conclusion

The effect of the principal factors controling the coagulation and syneresis are summarized in table 1. All the factors influencing milk coagulation also affect curd syneresis. However this influence may sometimes oppose the setting of the gel and the curd-whey separation.

Table 1: The effect of the principal factors on coagulation and syneresis.*

Physico-chemical factor	effect on the rate of coagulation	effect on firmness of curd	effect on the syneresis
Acidification of the milk	+	−	+
Addition of $CaCl_2$	+	+	−
Milk concentration by evaporation	+	+	+
Increase in amount of rennin added	+	0	+
Increase in temperature	+	−	+

* + = Increase
− = decrease
0 = without effect

The coagulation per se could be considered as the result of hydrophobic and electrostatic interreactions which compete in order to increase attractive forces between casein micelles and reduce the forces of repulsion. The calcium also interferes with the charges on the micelles and seems in no way to act as a bridgeing agent between them.

It must however be stated that the mechanism of the secondary phase has not yet been clearly elucidated. Thus when the structure of casein micelles is completely established a definite progress will have been achieved and coagulation and syneresis will become better understandable.

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