A C T A A L I M E N T A R I A P O L O N I C A
Vol. IV (XXVIII), No. 3 1978

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NON-DESTRUCTIVE VISCOMETRIC STUDIES OF ENZYMIC MILK COAGULATION
II. THE SECONDARY PHASE OF MILK COAGULATION

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Key words: milk coagulation, immobilized rennin

The ultrasonic viscometric method is applied to study the secondary phase of milk coagulation which has been initiated by immobilized rennin. The physico-chemical parameters for the secondary phase are evaluated.

INTRODUCTION

Enzymatic coagulation of milk can be divided into two phases: (a) the primary (enzymic) phase. during which the phenylalanyl-methionine bond of $\varkappa$-casein is attacked by the enzyme creating a metastable state of the micelle, and (b) the secondary phase, where the milk subsequently gels [1]. The secondary phase has not been studied in great detail since the separation of the two phases is not completely possible with soluble enzyme. However, use of immobilized enzymes allowed unique methods for studying enzymic milk clotting [2, 3]. Recently, immobilized pepsin has been succesfully applied as tool to study the secondary phase apart form the enzymic phase [4].

This paper reports the application of the ultrasonic viscometric method described in a previous paper [5] to characterize effects of dilution and temperature on the secondary phase of milk coagulation which has been initiated by immobilized rennin.

MATERIAL AND METHODS

The reconstituted fat-free dried milk (Instant) was used (12 g of the milk in 100 ml of 0.01 M $CaCl_2$) at pH 6.4. Calf fennin was immobilized on polyacrylamide gel by modyfication of the radiation procedure of

Kawachima and Umeda [6]. The insoluble enzyme preparation was washed thoroughly in distilled water in order to preclude any soluble enzymic activity in treated samples. The enzymatic reaction was carried out in a jacked stirred reactor adjusted between 15 and 35°C. The milk sample (10 to 50 ml) was treated with a weighted amount of the enzyme at vigorous stirring at the fixed contact time of 20 min. Aliquots of treated milk were then immediately transferred into a thermostated vessel and the viscosity change with time was recorded as previously described [5].

RESULTS AND DISCUSSION

To determine the effect of immobilized rennin concentration on the clotting time, different amounts of the enzyme were added to the milk sample in the enzyme reactor kept at 25°C, 30°C or 35°C, nad the enzymatic reaction was carried out for 20 min at vigorous stirring. To avoid coagulation of milk in the enzyme-reactor, the milk was separated from enzyme prior to the secondary phase. Samples of milk separated from the reactor were collected in a vessel thermostated at the same temperature as the enzyme-reactor. Clotting time (t_v) were then measured viscometricaly as previously described [5]. Plots of clotting time versus reciprocal enzyme concentration are shown in Fig. 1. Clotting time is related inver-

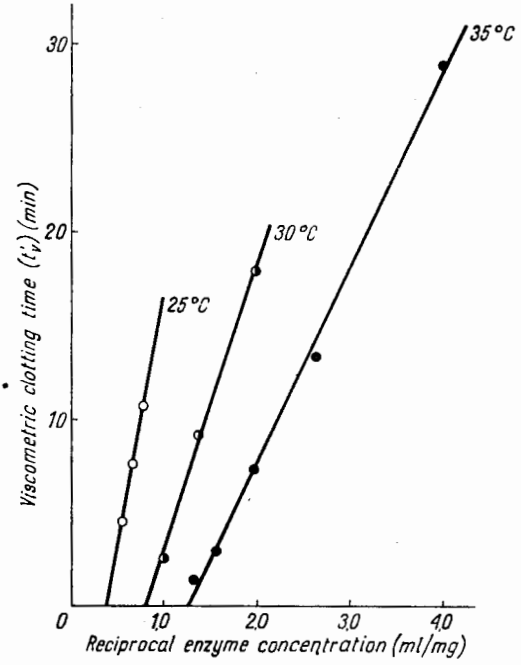

Fig. 1. Viscometric clotting time as related to reciprocal of immobilized rennin concentration in milk at temperature of: —○— 25°C, —◑— 30°C, —●— 35°C. Contact time = 20 min.

sely to the enzyme concentration as it has been also observed with soluble enzymes [5, 7]. To determine the effect of rennin on the secondary phase the rate of milk viscosity change was measured in the presence and in the absence of the enzyme during this phase. Data obtaining by varying the concentration of enzyme and thus changing the total clotting time, are presented in Fig. 2.

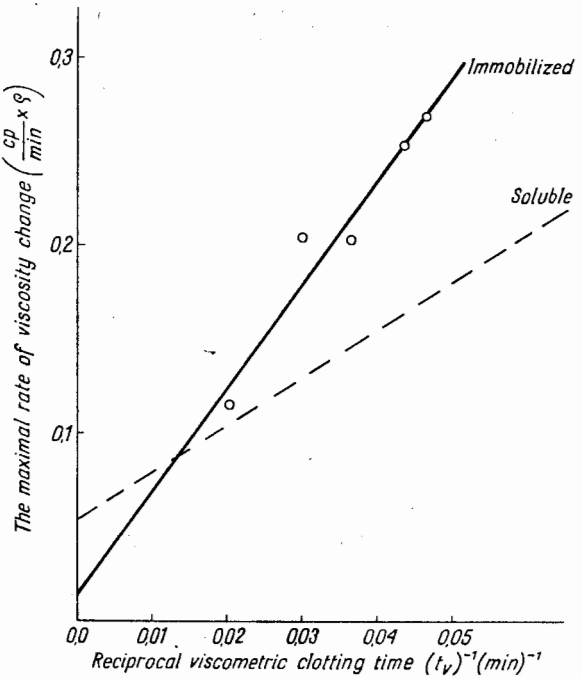

Fig. 2. The maximal rate of viscosity change of milk treated with immobilized (—) and soluble (— —) rennin at 35°C, as related to reciprocal clotting time. Data for soluble renning taken from a previous paper [5]

The linear dependence of the maximal rate of viscosity change during this phase on clotting activity (reciprocal of clotting time) are obtained whether immobilized or soluble rennin is used. However, the rate of viscosity change decreases when the enzyme is present during the secondary phase. One would expect faster gelation of milk treated with soluble enzyme, since the enzyme would still be present during the secondary phase and would influence the overall rate. The difference could result from the shift of the pH activity profile of rennin on immobilization [6, 8] or it could be due to adsorption of some milk fractions on the enzyme support [4, 9]. Since pH effects drastically both the primary phase and secondary phase of milk coagulation the pH dependence of the rate of gelation are yet to be evaluated.

EFFECT OF DILUTION

The effect of diluting milk from the enzyme-reactor with untreated milk and with distilled water on the clotting time is shown in Fig. 3. The enzymic reaction was carried out at 35°C. All samples of treated milk had the same contact time with immobilized rennin (20 min) and the same enzyme to milk ratio (0.5 mg of enzyme per ml of milk). Aliquots of the treated milk were dispensed into premeasured amounts of diluent in a thermostated vessel prewarmed at 35°C. As is shown in Fig. 3 clotting

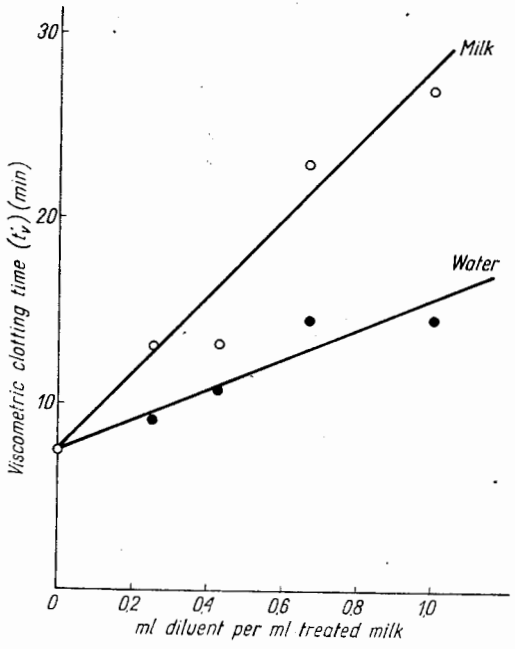

Fig. 3. Effect of dilution of milk treated with immobilized rennin on clotting time at 35°C. Diluents were: ● water, ○ untreated milk. Enzyme to milk ration = 0,5 mg of enzyme per ml of milk.

times increase with increasing additions of fresh milk but adding the same amounts of distilled water has less effect on the clotting time. Similar effects of dilution on the clotting time have been recently reported for immobilized pepsin [4]. Our studies of the milk viscosity revealed that dilution at the rennin-treated milk influenced also the rate of viscosity change during the secondary phase. The effect at diluting the rennin-treated milk with fresh milk and with water on the maximal rate of viscosity change during the secondary phase, is shown in Fig. 4. Since the contact time for all samples in these experiments was the same, presumably the same number of the rennin sensitive bonds were broken in each sample. Thus the percentage of bonds broken in the mixture would de-

crease proportionally with increasing dilution with water or with fresh milk.

In the case of dilution with water, as shown in Fig. 4, the rate of viscosity change is proportional to the concentration of rennin treated

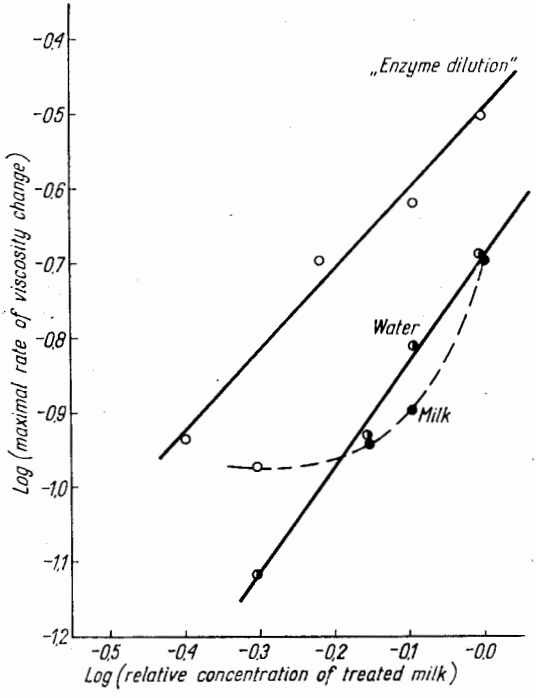

Fig. 4. Effect of dilution of milk treated with immobilized rennin on the maximal rate of viscosity change at 35°C.
Diluents were ◑ — water, ● — untreated milk. Enzyme to milk ratio = 0,5 mg of enzyme per ml of milk. ○ — dilution of enzyme in the treated milk.

micelles. However, in the case of dilution with fresh milk such relation is not obtained, and suggests interference in clotting by unreacted micelles. The concentration of treated micelles was also varied by changing the enzyme to milk ratio in the reaction mixture. In that case, the rate of viscosity change during the secondary phase appeares also to be proportional to the concentration of the rennin-treated micelles (Fig. 4). One can assume that the maximal rate of viscosity change during the secondary phase is directly proportional to the initial rate of the process of milk gelation. Thus, the order of the process, n, with respect to the concentration of rennin-treated micelles, can be obtained from the slope of a graph of the logarithmus of the maximal rate plotted against the logarithmus of the relative concentration of the treated milk (the initial rate method for determining order of reaction). The plots in Fig. 4 sug-

gest the order of 1 (1.06 ± 0.35) and 1.5 (1.43 ± 0.32) when the concentration of rennin-treated micelles is varied by the change in amount of enzyme used and by dilution with water, respectively. It has been suggested [10] that during enzyme treatment of milk para — κ-casein is formed, which subsequently forms bridges interlinking micelles to produce gelatinous coagulum. To account for the interference in the rate of the gel formation by unreacted micelles one can assume that also an additional specific interaction occurs between micellar components other then or in addition to para — κ-casein. Untreated micelles could be also entrapped in the gel.

TEMPERATURE EFFECT

The effect of lowering the temperature on retarding the formation of the clot is well known. Recent studies of the secondary phase with pepsin-glass indicate that the clotting time has a characteristic very high temperature coefficient (Q_{10}) of 12, [4] although lower than those reported when using soluble enzymes [11]. By varying the temperature of only

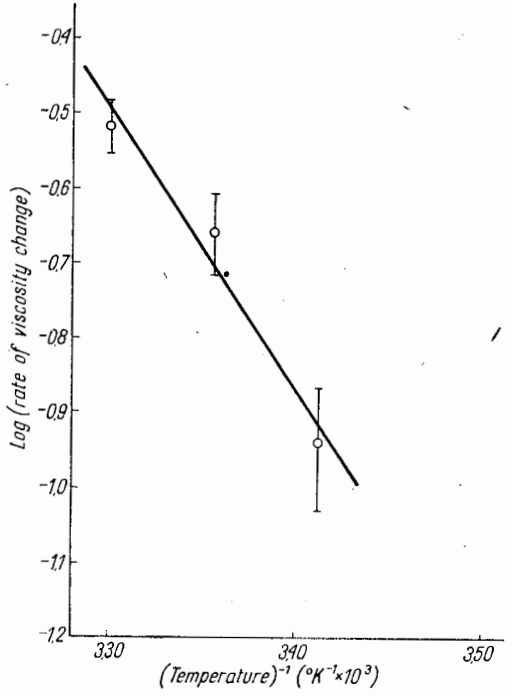

Fig. 5. Effect of temperature of the secondary phase on the maximal rate of viscosity change. Enzymic phase temperature = 15°C.
Enzyme to milk ratio = 1,5 mg of enzyme per ml of milk, at the contact time of 20 min.

the secondary phase it was possible to determine the effect of temperature on the maximal rate of viscosity change during this phase. Data presented in Fig. 5 indicate a high sensitivity of the rate of milk gelation to temperature. All samles has the same enzyme to milk ratio of 1.5 mg of enzyme per ml of milk and the same contact time (20 min) at 15°C. By lowering the temperature of the enzyme-reactor to 15°C it is possible to retain sufficient enzyme activity and prevent coagulation of the milk in the reactor. Since the concentration of rennin-treated micelles is the same for all samples, the maximal rate of viscosity change is directly proportional to the rate constant of the process of milk gelation. Thus the graph in Fig. 5 represents the simple Arrhenius plot for the process.

Based on this plot, activation energy value of 72 kJ/mol (17 kcal/mole) for the 20° to 30°C range has been obtained. This yields the value of enthalpy of activation of 69 kJ/mole (16,5 kcal/mole) at 25°C. The value of enthalpy of activation for the overall process could be consistent with the idea of milk coagulation being basically a charge — neutralization process [12, 13] if only the energies of the elementary reactions for the process became available [14]. This requires studies on the effect of pH on the enthalpy of activation of the process.

CONCLUSIONS

1. Clotting time as determined viscometrically for the secondary phase of milk coagulation is inversely related to the immobilized rennin concentration, between 25 and 35°C, and this dependence can be used to determine clotting activities of immobilized enzyme preparations.

2. The analyses of the effect of dilution of treated milk on the clotting times and on the rate of viscosity change during the secondary phase indicate interference in the clotting process by unreacted casein micelles.

3. The linear dependence of the maximal rate of milk viscosity change during the secondary phase on the concentration of rennin-treated milk yields the order of 1 to 1,5 for the process of milk gelation.

4. The dependence of the maximal rate of viscosity change on temperature allows to estimate the enthalpy of activation for the overall process of milk gelation. The value of the enthalpy of 69 kJ/mole (16,5 kcal/mole) at 25°C could be consistent with the idea of milk coagulation being a charge — neutralization process.

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Manuscript received: August, 1977.
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NIE NISZCZĄCE BADANIA WISKOZYMETRYCZNE ENZYMATYCZNEGO PROCESU KRZEPNIĘCIA MLEKA. II. WTÓRNA FAZA PROCESU KRZEPNIĘCIA MLEKA

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Streszczenie

Do badania fazy wtórnej enzymatycznego procesu krzepnięcia mleka zastosowano wiskozymetr ultradźwiękowy. Do zainicjowania procesu krzepnięcia użyto preparatu unieruchomionej renniny. Wykazano, że występuje liniowa zależność czasu krzepnięcia wyznaczanego wiskozymetrycznie od zdrowotności stężenia unieruchomionego enzymu. Szybkość zmiany lepkości mleka w fazie wtórnej była wprost proporcjonalna do aktywności koagulacyjnej tego enzymu. Mleko, które przereagowało z enzymem poddawano rozcieńczeniu i zmianom temperatury badając wpływ tych czynników na czas strącania skrzepu i szybkość procesu żelowania. Wykazano udział nieuszkodzonych enzymatycznie micelli w procesie wytwarzania skrzepu. Określono rząd procesu żelowania mleka na $n = 1$ do 1,5 w stosunku do stężenia miceli poddanych działaniu enzymu oraz wartość entalpii aktywacji tego procesu równą 69 kJ(mol) 16,5 kcal(mol) w 25°C.