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NON-DESTRUCTIVE VISCOMETRIC STUDIES OF ENZYMIC MILK COAGULATION

I. AN ULTRASONIC VISCOMETRIC METHOD FOR THE MEASUREMENT OF MILK CLOTTING ACTIVITY OF PROTEOLYTIC ENZYMES

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Key words: ultrasonic viscometry, milk coagulation, proteolytic activity.

An ultrasonic viscometry method has been applied to study enzymic milk coagulation. Time elapsing to the starting of the change of milk viscosity as caused by proteolytic enzyme is directly correlated to reciprocal enzyme concentration and the rate of the change stands in close correlation to enzyme concentration. The method based on these correlations may be used to determine milk clotting activity and to establish the suitability of enzymes for cheese-making.

INTRODUCTION

At present, the milk clotting activity of rennin and rennin-like enzymes or commercial rennets is assayed by measuring the time required to clot a suitable substrate and the method is based on the visual observation of coagulation [1].

In a number of papers [2, 3], attemps have been made to provide a viscometric method for use in the assays, taking advantage of the fact that the viscosity of milk or casein solution undergoes considerable changes during the action of the enzyme on the substrate.

In our studies of the effect of ionizing radiation on rennin [4] several methods have been taken into account in order to provide an objective method for use in the investigation of both the milk clotting activity and specificity of the enzyme with respect to the substrate. The present paper discusses an ultrasonic viscometry method applied in studies of enzymic milk coagulation.

MATERIAL AND METHODS

All experiments were done on reconstituted fat-free instant dried milk (produced in this country). The milk sample was prepared by dissolving 12 g of milk in 100 ml of 0.01 M $CaCl_2$ followed by filtration of the

resulting solution to remove any undissolved material. The enzymes used were:

— calf rennin, a liophylized enzyme obtained after purification of a commercial preparation (Bacutil) by dialysis,

— calf rennin, a technical preparation produced by Koch-Light Lab. Ltd (England),

— pepsin, a commercial preparation produced by Bacutil, of a proteolytic activity of 2.78 PU^{IIb} [5],

— papain, a crude preparation produced by Loba-Chemie (Austria).

Rennin was dissolved in distilled water, pepsin in 1 mM HCl and papain in a solution containing 1 mM EDTA and 5 mM cystein.

The viscosity of milk was measured with a Unipan type 508 ultrasonic viscometer provided with an Unipan type 505-2 probe. The output voltage was recorded with a G1B1 compensating type recorder. The variable measured is a product of dynamic viscosity η and density ϱ of the liquid, and the variable unit is 1 cP $\times$ g/cm^3. The ultrasonic probe was immersed in 50 ml of milk (pH 6.4) thermostated at 35°C in a glass vessel. At constant output signal recorded, a small portion (0.05-0.5 ml) of the enzyme solution was added to the milk sample followed by complete mixing during 30 secs.

RESULTS AND DISCUSSION

The change of the output voltage with time of enzymatic reaction, as expressed in percent of the recorder scale — P, is shown in Fig. 1 (curve 1). The variable $\eta\varrho$ for milk expressed in cP$\times$g/cm^3 is related to the value of P as follows:

$$\eta\varrho = \frac{P^2}{200}$$

The change of the variable $\eta\varrho$ in the course of enzymatic reaction is shown in Fig. 1 (curve 2). A constant value of $\eta\varrho$ is obtained in the initial period of the reaction. A rapid increase of $\eta\varrho$ has been observed in a certain time range, and the rate of the change exhibits a maximal value at the time t_m (Fig. 1, curve 3). It can be tentatively assumed in the case of milk that the variable $\eta\varrho$ yields values very close to the values of viscosity η. It has been found that the increase of viscosity of enzyme-treated milk and the clotting of milk occur simultaneously. The time when viscosity begins to increase depends on the enzyme concentration. The changes of the output voltage as caused by rennin acting on milk at various enzyme concentrations are shown in Fig. 2.

By drawing a tangent to the curve through its point of inflexion, it is possible to determine an extrapolated time of the start of viscosity

increase — t_v which corresponds to the point of intersection of the tangent and the time axis. The time axis is led through the point corresponding to the initial output voltage value. The value of time t_v is a characteristic parameter of the enzymatic process. In order to test the effect of the ultrasonic probe on milk coagulation, the viscometer was disconnected for a variable period of the enzymatic reaction. As shown in Fig. 3, both

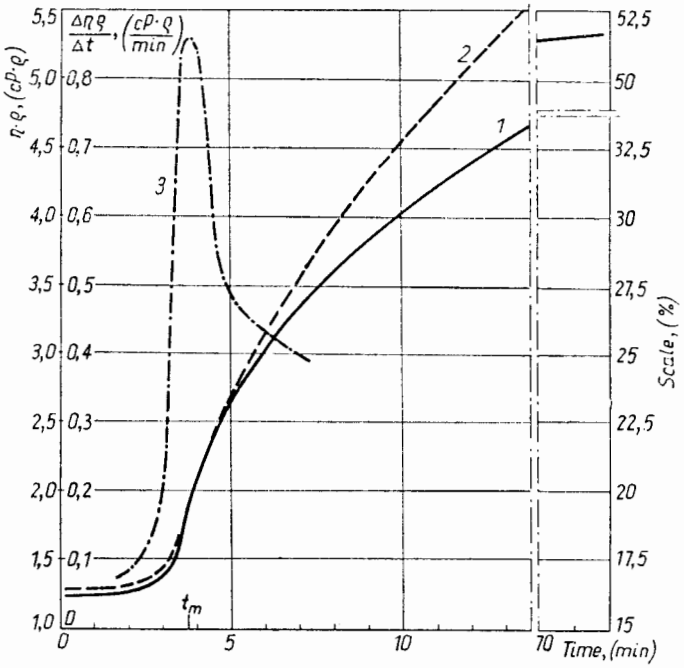

Fig. 1. Changes of output voltage; 1 — calculated variable $\eta\varrho$, 2 — the rate of the variable $\eta\varrho$ increase, 3 — with time of rennin-treated milk at 35°C; enzyme concentration: 0.015 g dm^{-3}

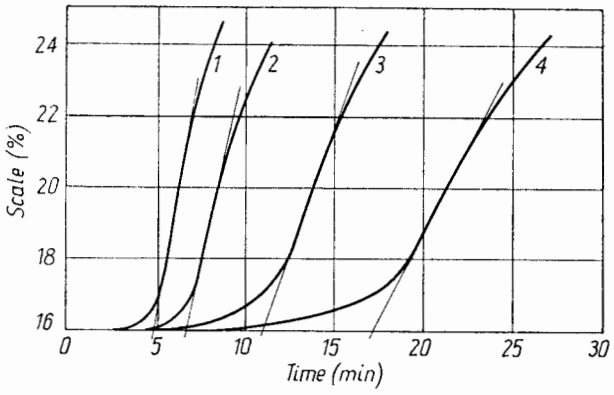

Fig. 2. Changes of output voltage from ultrasonic viscometer caused by renning acting on milk at 35°C, for different enzyme concentrations; 1 — 0.01 g dm^{-3}, 2 — 0.007 g dm^{-3}, 3 — 0.004 g dm^{-3}, 4 — 0.0025 g dm^{-3}

the values of time t_v and the shape of the curves remain unchanged within the limits of the experimental error. It seems very likely that the action of the ultrasonic probe does not influence the coagulation process.

The earlier experiments [2, 3] showed an initial fall in viscosity of enzyme-treated milk, followed by a rise. It is assumed that this initial decrease in viscosity is due to a decomposition of a part of casein micelles, followed in later stages by the formation, of aggregates which increase viscosity, and then, of a rigid gel structure. The aggregation stage can be disturbed and the gel structure destroyed when a drastic method of measuring viscosity is applied, as in the case of capillary, rotating or sinker viscometers. No change of viscosity has occured in milk containing rennet type animal or microbial enzyme when measured by Vàmos et al. [6] with a Rheomat rotating viscometer, although in the case of other proteolytic enzymes an increase of viscosity was observed with simultaneous precipitation of milk.

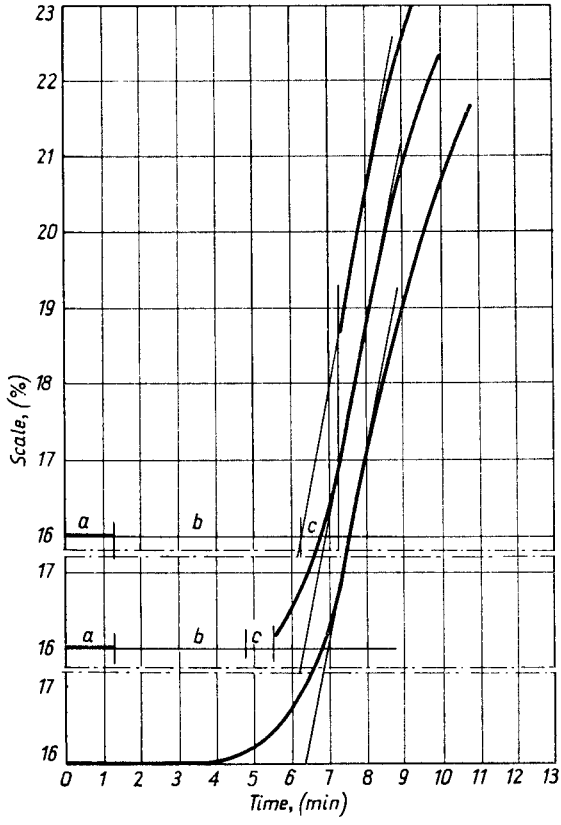

Fig. 3. Changes of output voltage from ultrasonic viscometer with time recorded at continuous or discontinuous conditions of measurement for samples with identical rennin-to-milk ratio; a — recording with connection of viscometer, b — disconnection of viscometer, c — subsequent connection of viscometer with no recording

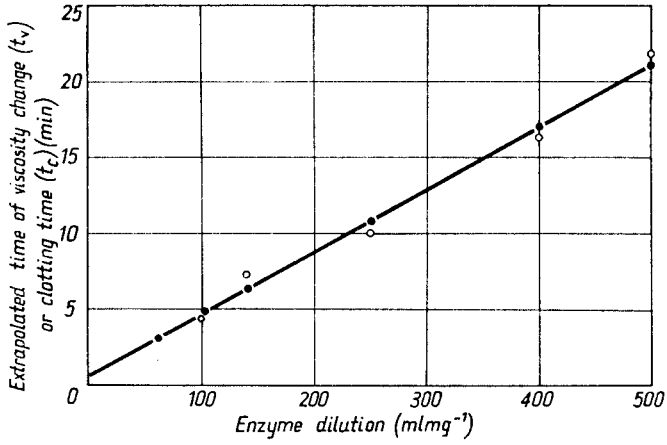

Fig. 4. The extrapolated time elapsing to the starting of milk viscosity (t_v) as related to rennin dilution in milk at 35°C (—●—). The open circles denote the cloting time (t_c) determined for comparable samples (—○—)

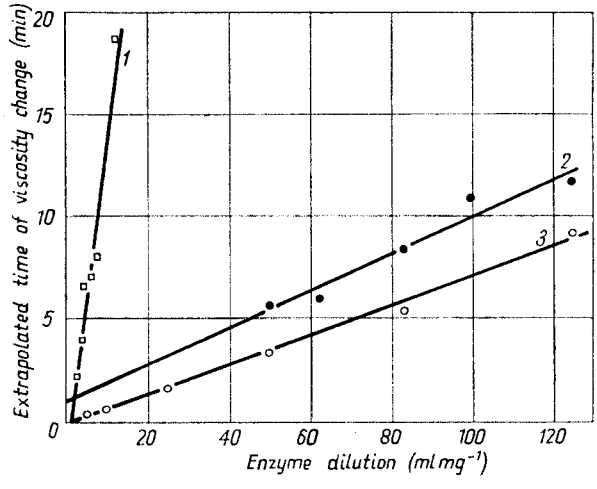

Fig. 5. The extrapolated time elapsing to the starting of milk viscosity increase (t_v) as related to enzyme dilution in milk at 35°C, in the case of: 1 — papain, 2 — rennin *Koch*-Light, 3 — pepsin

The shape of the curves reflecting the change of milk viscosity obtained by our method enables to distinguish three phases in enzymic milk coagulation. However, during the enzymatic decomposition of casein micelles, in the initial period of the process, no fall in viscosity was observed since the change is presumably too low as compared to the sensitivity of the method.

The rapid increase of viscosity reflects the process of clotting, and the further increase is a result of a structural change in the coagulum. It has

been found that the extrapolated time of the rapid change of viscosity (t_v) is a linear function of enzyme dilution. Moreover, the values of time t_v approximate the values of the clotting time (t_c) which has been determined by a classical method [1] in parallel experiments (Fig. 4). The linear dependence of time t_v on the enzyme dilution provides the possibility of using it for the determination of the milk clotting activity of enzymes. This assumption has been tested for a number of proteolytic enzymes and a good correlation between t_v and reciprocal enzyme concentration has been obtained in the t_v range from fractions of a minute to about 20 minutes (Fig. 4 and 5). It has been also found that the

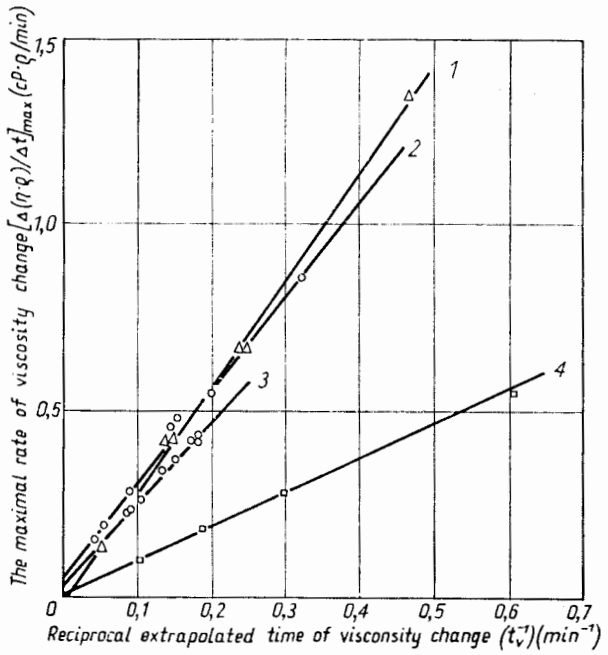

Fig. 6. The dependence of the maximal rate of the change of milk viscosity on reciprocal extrapolated time t_v for milk treated at 35°C with: 1 — papain, 2 — rennin, 3 — rennin Koch-Light, 4 — pepsin

maximal rate of viscosity change is linearily related to enzyme concentration which can be normalized for different preparations by replacing it with the reciprocal t_v (Fig. 6). Table shows regression equations representing the tested relationships. The correlation coefficients shown in the Table clearly ponit to a very close correlation between the points of the regression line and the measured values. The slopes of the regression lines, as expressed numerically by the regression coefficients, and representing the maximal rate of viscosity change caused by an enzyme unit, did not differ significantly in the case of rennin prepara-

tions. With the preparations of rennin on one hand, and of pepsin and papain on the other, the difference was significant at a probability level of 95%. The difference between enzymes is probably due to the fact that the enzymes behave differently in milk in creating a metastable state of casein micelles which subsequently aggregate. Thus the course of milk coagulation and the physical properties of the gel formed should depend on the enzyme used.

Table. Linear regression equations: $y = ax+b$ describing the relationship between characteristic parameters of the process of enzymic milk coagulation

Enzyme	t_v (min) as a function of reciprocal enzyme concentration (ml mg^{-1})			$\left[\frac{\Delta(\eta\varrho)}{\Delta t}\right]_{max.}$ $\left(\frac{cP}{min}\varrho\right)$ as a function of $\frac{1}{t_v}$ (min^{-1})		
	regression equation	r	Δa	regression equation	r	Δa
Rennin	$y = 0.041x+0.70$	0.9996	0.0015	$y = 2.54x+0.05$	0.996	0.25
Rennin (Koch-Light)	$y = 0.089x+1.01$	0.972	0.039	$y = 2.15x+0.04$	0.995	0.20
Pepsin	$y = 0.072x-0.14$	0.997	0.008	$y = 0.88x+0.02$	0.999	0.04
Papain	$y = 1.560x-2.26$	0.965	0.59	$y = 2.89x-0.02$	0.998	0.28

r — correlation coefficient, Δa — standard error of the term a at the probability level of 95%.

The increasing use of renning substitutes for processing of the curd made it necessary to develop an objective method for the examination of the technological quality of the enzymes. Our studies have shown that the properties of some proteolytic enzymes in their action on milk could be recognized by an analysis of the rate of change of milk viscosity. Thus the viscometric method presented here may be used also to establish the suitability of enzymes for cheese-making.

CONCLUSIONS

1. The method developed for use with an ultrasonic viscometer, enables characterization of enzymic milk coagulation by a continuous monitoring of milk viscosity without disturbing any phase of the process.

2. The method based on the correlation between the reciprocal enzyme concentration and the extrapolated time elapsing till the starting of the viscosity increase may be used to measure curdling activity of both rennet type enzymes and other proteolytic enzymes.

3. The maximal rate of the change of viscosity is directly proportional to enzyme concentration. The comparative analysis of such correlations

may be used as a preliminary method to establish the suitability of enzymes for cheese-making.

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NIE NISZCZACE BADANIA WISKOZYMETRYCZNE ENZYMATYCZNEGO PROCESU KRZEPNIĘCIA MLEKA

I. WISKOZYMETRYCZNA METODA OZNACZANIA AKTYWNOŚCI KOAGULACYJNEJ ENZYMÓW PROTEOLITYCZNYCH

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Streszczenie

Do badania enzymatycznego procesu krzepnięcia mleka zastosowano wiskozymetr ultradźwiękowy. Na podstawie opracowanej metody pomiaru lepkości mleka poddanego działaniu enzymów proteolitycznych, jak: rennina, pepsyna lub papaina, wykazano obecność trzech faz w procesie krzepnięcia: enzymatycznej, krzepnięcia właściwego, zmian strukturalnych powstałego skrzepu. Ponadto wykazano, że poszczególne fazy procesu krzepnięcia nie są zakłócane podczas pomiaru lepkości.

Zaproponowano metodę określania aktywności koagulacyjnej renniny i innych enzymów proteolitycznych, polegającą na wykorzystaniu prostoliniowej zależności ekstrapolowanego czasu początku wzrostu lepkości mleka od odwrotności stężenia obecnego w nim enzymu. Na podstawie analizy porównawczej wpływu stężenia enzymu na szybkość zmiany lepkości krzepnącego mleka wykazano istnienie różnic w specyficzności działania koagulacyjnego badanych enzymów. Uzyskane wyniki pozwalają wnosić, że zastosowana metoda wiskozymetryczna może być wykorzystana do ustalania przydatności enzymów do celów serowarskich.