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A Simple Method for Determining Activity of Milk Clotting Enzymes

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응유효소의 간편한 역가측정법

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Abstract

The process of milk clotting by enzymes was confirmed to be consisted of two distinct stages: an enzymic coagulation stage followed by a nonenzymic clotting stage. The endpoint of the enzymic stage was determined simply by measuring flow distance of milk-enzyme system in a regular test tube being laid down to five degree slope after the reaction. By measuring the time elapsed during the enzymic stage a new method of evaluating the power of milk clotting enzymes was proposed.

Introduction

Milk clotting enzymes are essential agents for cheese industry. Inspite of the great importance from viewpoints of scientific, technological, and commerical, an ideal method for measuring milk clotting activity of enzymes has never been established. The problem of observing accurately the endpoint of clotting is one of the main reasons for the difficulty[4].

Berridge[2] developed a convenient apparatus in which the sample and rennet are placed in a tube, which is rotated at an angle in a thermostatically controlled bath. With such an apparatus, the formation of flocs in the film of milk on the inner surface of the tube is observed. Arima et al.[1] used a smaller tube filled with dilute red ink inserted into the larger test tube having reaction mixture in it. The time elapsing between mixing of reagents and the first appearance of solid material against the red-dye background is measured.

A different method for measuring enzyme activity was proposed by Scott-Blair and Burnett.[9] The flow of rennetted milk through

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a capillary was measured and the detection of curdling was indicated by a change in the slope of the curve of flow rate. Richardson et al[8], used rotational type viscometers to measure curd firmness continuously and tried to indicate the transition at the coagulation zone clearly. The changing point, from the first-stage to the second-stage of the milk curdling reaction, was more clearly demonstrated by Foltman[5] and by Kopelman and Cogan[7] through continuous monitoring of the viscosity of a milk-enzyme system. These authors proposed separately equations in which the time span for the overall curdling reaction was indicated with both factors involved in the first and second stages of the reaction.

From the present study it was confirmed that the first stage of the milk curdling reaction was enzymic and the second stage was non-enzymic. Based on this discovery the time needed for the first stage reaction of rennet was determined simply with regular test tubes and proposed a calibration curve with which power of milk clotting enzymes can be evaluated.

Materials and Methods

1. Substrate and enzymes

eleven per cent reconstituted milk as the substrate was prepared by dissolving skim milk (Difco) in 0.05 M phosphate buffer (pH 6.5) and calcium chloride (CaCl) was added to the level of 0.01 M. Calf rennet was purchased from Nutritional Biochemical Co. (NBC).

2. Milk flow distance

A test tube of 2.2 cm in diameter and 19 cm in length was graduated up to 16'cm from the bottom. Into the tube five ml of the reconstituted milk was transferred and prewarmed for 15 minutes in 35°C water bath. Reaction was started by adding 0.5 ml of enzyme solution having different dosages to the reconstituted milk and kept the tubes standing upright on a rack in the same water bath. During the reaction proceeds test tubes were taken out from the water occationally to read the distance of the milk movement. First of all the tubes should be laid down to 85 degree from the upright position as shown in Figure 1. This was done converiently by laying the whole test tube rack on a boad fixed to 5 slope. The five ml milk in the tube flowed along the test tube wall to reach certain distance from the botton of the tube and stopped. Then the distance was read.

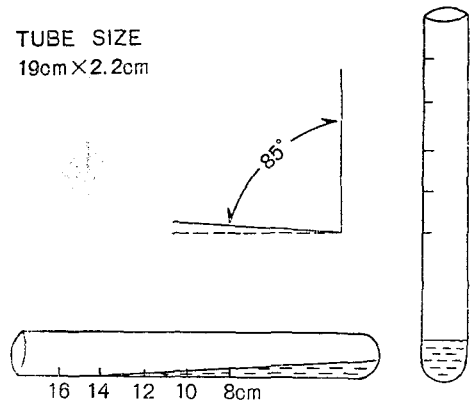

Fig. 1. A Schematic Diagram Showing the Procedure of Determining Flow Distance of Milk-Enzyme System. The reaction mixture flowed to about 14 cm distance from the tube bottom when the tube was laid down to 5° slope after certain period of reaction.

3. Milk viscosity

The viscosity of the milk was measured using a viscometer supplied by Brookfield Engineering Co. (Synchro-Lectric Model LVT). Three hundred millimiters of milk were ttransferred to a 500 ml glass beaker and prewarmed for 15 minutes in a 35°C water bath. No. 2 spindle running at 100 rpm was lowered down. The enzyme at concentrations equivalent to the tube test was added and readings were taken at 30 second intervals.

Results

A typical two-stage reaction occuring during the process of curdling milk with enzyme is shown in Figure 2. When five ml of reconstituted milk was treated with 0.5 mg of calf rennet at 35°C, there was no change in the liquidity of the milk for about four minutes. This was shown by the constant flow distance of 16 cm in the Figure. As the reaction time exceeded four minutes, a sudden decrease in the milk liquidity occured and the distance of the milk flow in the tube reduced accordingly. By a perpendicular line drown from the intersection "A" of constant and steep decrease to "B" on the abscissa, the enzymic curdling phenomenon could be devided into two stages. The first stage may be designated as pre-clotting or coagulation stage and an actual clotting takes place at the second stage.

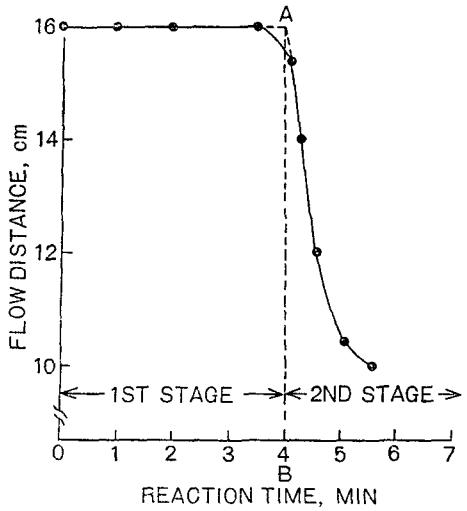

Fig. 2. Double-Stage Phenomenon Observed During the Process of Milk Curdling by Rennet. Five ml of the reconstituted milk was treated with 0.4 mg of NBC calf-rennet at 35°C with periodical readings for flow distances.

Figure 3 shows the effect of enzyme concentrations on the time to complete the first-stage of the milk curdling reaction. The duration of the first-stage reaction increased with the

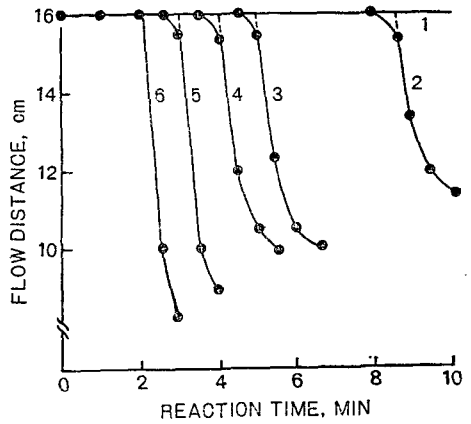

Fig. 3. Effect of Enzyme Concentration on the Duration of the First-Stage Reaction Determined by Measuring the Flow Distance of Reaction Mixture. Numericals show the amounts of rennet added to 5 ml of the substrate: 1; 0.1 mg, 2; 0.2 mg, 3; 0.3 mg, 4;0.4 mg, 5; 0.5 mg, and 6; 0.75 mg.

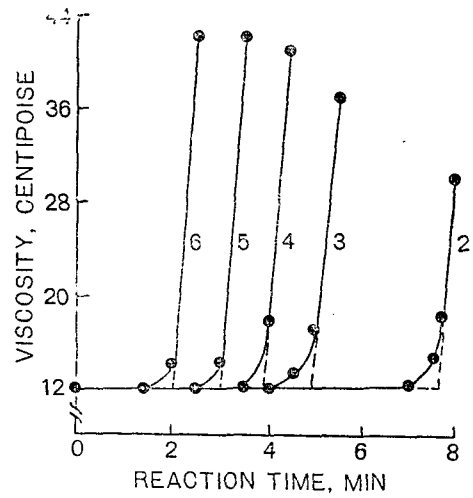

Fig. 4. Effect of Enzyme Concentration on the Duration of the First Stage Reaction Determined by Measuring Viscosity of the Reaction Mixture. Various amounts of NBC calf rennet were added to 300 ml-substrates to make concentrations equivalent to those of Figure 3 indicated by the same series of numericals.

decrease of the enzyme concentration. During the second-stage reaction of each enzyme concentration the curve looses its linearity as the reaction proceeds. But this is merely due to the disadvantage of using test tubes and does not mean the decrease of clotting speed. The fact is clearly shown in Figure 4 where viscosity increased linearly in all cases of enzyme concentrations. The straight lines are parallel each other suggesting that the speed of reaction of the second-stage is not dependent to the concentration of the enzyme.

The time values for the first-stage reactions obtained through the curves of Figure 3 were plotted against the reciprocals of the corresponding enzyme concentrations and shown in Figure 5. Apparently the duration of the first-stage reaction of rennet in milk is reversely proportional to the concentration of the enzyme. The high correlation coefficient of 0.998 suggests a reliable use of the straight line as calibration curve for determining the activity of milk clotting enzymes.

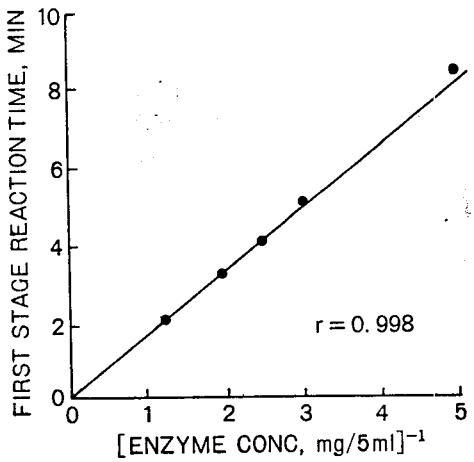

Fig. 5. A Calibration Curve Composed of the Time Needed to Complete the Enzymic First Stage Reaction against the Reciprocal of Enzyme Concentration. Data were obtained from the results of Figure 3. r: correlation coefficient.

Discussion

The process of milk curdling by enzymes is thought to be consisted of two stages. Jenness and Patton[3] used terms of coagulation and clotting for the two stages. The former refers to precipitation in the form of loose separate flocs, and the latter denotes the formation of a smooth gel or clot occupying the entire volume originally occupied by the milk.

Apparently the first stage of the reaction is enzymic and the second stage is of non-enzymic. This is proved by the results shown in Figure 4 where the change of milk viscosity during the reaction is plotted against time. The time to complete the first stage of reaction is dependent to the concentration of enzyme which is characteristic for an enzyme reaction. However, the straight parallel pattern of the second stage curves for defferent enzyme concentrations suggests that the speed of milk clotting is independent to the concentration of enzyme. Therefore, the second stage of the milk curdling is not an enzymic reaction. Since the second stage of the milk curdling reaction is non-enzymic one, we may omitt it from the evaluation of enzyme activity. The end point of the first stage reaction can be determined precicely by using a viscometer or by the method developed in the present investigation. Then the power of the enzyme can be clearly evaluated by measuring the time from mixing until the end of the first stage reaction of milk curdling. The use of sophisticated viscometer may give more accurate determination, but the requirement of smaller amount of sample and simplicity may grant advantages to the test tube method.

요 약

효소에 의한 우유의 응고과정은 효소적인 단계와 비효소적인 단계의 두 가지로 구성되어 있다는 사실이 확인되었다. 효소적인 단계가 끝나는 점을

시험관속에서 반응을 시킨 다음 그 시험관을 5도의 경사까지 기울었을 때 우유가 시험관 벽을 타고 흐를수 있는 거리를 가지고 측정하였다. 효소적인 단계에 소요되는 반응시간을 측정하므로서 응유효소의 역가를 평가하는 새로운 방법이 제시되었다.

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