A METHOD FOR DETERMINING THE RENNET ACTIVITY OF CHYMO-TRYPSIN

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When pepsin is added to milk a gradual rise in viscosity takes place until the milk begins to clot. A quantitative expression for the rennet activity of pepsin can thus be given in terms of the increase in viscosity of the milk solution (1). The viscosity method is sufficiently precise and can be conveniently used in all cases of clotting of milk or blood where the visible clotting follows a gradual increase in the viscosity of the fluid.

When chymo-trypsin is added to milk a slight, gradual drop in the viscosity of the milk takes place; there is no increase in viscosity until the beginning of clotting when the viscosity rises very rapidly and the solution suddenly sets to a solid gel. This is shown in Fig. 1.

The abrupt solidification of milk by chymo-trypsin enables the exact time of clotting to be conveniently determined. The various methods described in the literature for measuring the clotting of milk or blood (2) are either very complicated and time consuming or are not precise. The following method, used during a long series of investigations, was found to be quite accurate and very simple in operation. The method is based on the fact that when a concentrated dried milk powder solution, to which chymo-trypsin has been added, is allowed to flow slowly through a narrow tube the uniform flow of the milk is either brought to a sudden stop when the milk clots or, if the clot is soft, a definite mark of curd is left on the walls of the tube even after the clotted milk has continued to flow slowly through the tube. The volume of milk which escapes from the tube before clotting occurs can thus be determined. If, in addition, the rate of flow of the milk in the tube is known then the time required for the milk to clot can be readily calculated.

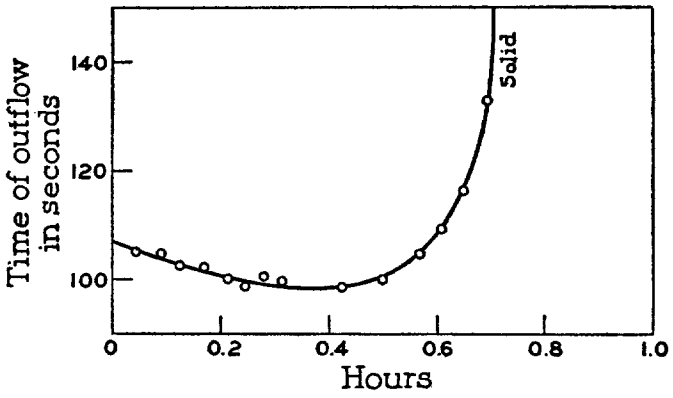

FIG. 1. Effect of chymo-trypsin on viscosity of 10 per cent solution of Klim milk pH 5.0 at 35.5°C.

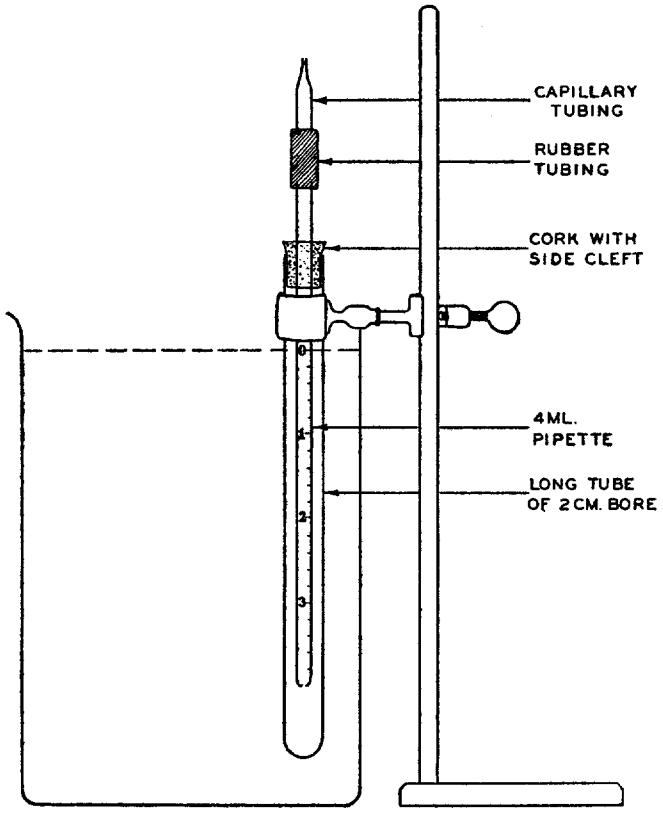

FIG. 2. Apparatus for measuring the rennet activity of chymo-trypsin.

Apparatus

The apparatus used is similar to that described by Heubner and Rona (3) for blood clotting but is much simpler in construction. It consists of the following parts, as shown in Fig. 2.

1. A straight pipette of 4 ml. content, graduated to 0.1 ml. It is made from an ordinary 5 ml. measuring pipette by cutting off the tip at the 4.1 ml. mark and then fusing the end to a bore of about 1 mm. in diameter. The graduations are, as usual, from 0 downwards. The marks are rubbed in with a black glass pencil to make them more conspicuous when the pipette is filled with milk.

2. A glass tube about 30 cm. long and 2 cm. inner bore. The tube is clamped in a vertical position in a constant temperature bath with transparent walls.

3. A number of short pieces of glass tubing about 3 mm. in diameter drawn out to very fine capillaries.

Milk Solution

20 gm. Klim powdered milk is ground up gradually to a creamy paste in a large mortar by the addition of increasing amounts of distilled water, then washed into a 100 ml. volumetric flask containing 10 ml. of M/1 sterile acetate buffer pH 5.0. The solution is made up to mark with water and filtered through gauze. The milk, after addition of toluene can be stored for 2 or 3 weeks at 5°C. without any significant change in its behavior.

Operation

1 ml. enzyme in water or in M/10 acetate buffer pH 5.0 is blown into a test tube containing 10 ml. milk at 35.5°C. and the tube is stirred immediately. A stop-watch is started at the moment of addition of the enzyme. The milk is drawn up to the zero mark of the 4 ml. pipette by means of a short piece of rubber tubing fitted on the pipette and provided with a spring clamp. When the milk has reached the zero mark the tubing is clamped and a suitable capillary tubing is inserted in the rubber tubing. The spring clamp is removed from the rubber tubing while the tip of the pipette is still in the milk. This brings the level of the milk slightly above the zero mark. The pipette is transferred into the long glass tube

in the water bath and fixed in position. The time required for the first 0.5 ml. of milk to flow out is then determined by means of a second stop-watch. The first stop-watch is stopped immediately after starting the second watch. The reading on the first watch indicates the time elapsed between the moment of mixing the enzyme with the milk and the moment when the milk passed the zero mark in the pipette during its continuous flow.

The pipette is left in the bath until the milk clots. Observation is made afterwards of the height of the clotted milk in the pipette. This observation can be made any time after clotting and it is unnecessary to watch the experiment. If the clot is soft it will continue to flow but there is always a distinct curd left on the wall of the pipette which shows where clotting began.

The method is convenient as well as rapid. A large number of pipettes can be started one after another and left alone in the bath for final reading.

It is advisable to use capillaries of such dimensions that the first 0.5 ml. requires 3 to 5 minutes to flow. Such concentrations of enzyme should be selected that clotting occurs within 10 to 20 minutes after addition of the enzyme to the milk solution.

Calculations

It was mentioned above that in the case of clotting of milk by chymo-trypsin the viscosity of the milk is not changed appreciably until the moment of clotting. The rate of the flow of milk depends, then, on the size of the capillary on the top of the pipette as well as on the opening at the outflow, and is proportional to the height of the milk in the pipette; *i.e.*,

$$-\frac{dh}{dt} = Kh \qquad \text{(Equation 1)}$$

Hence, the time required for the outflow of any volume of milk from the level h_o to level h_t is

$$t = \frac{2.3}{K} \log \frac{h_0}{h_t} \qquad \text{(Equation 2)}$$

Since the pipettes are graduated from zero downwards the original height h_o of the milk is 4.0 and the height h_t at any time t during

flowing is 4.0 minus the graduation mark on the pipette at the level of the milk. The time required for the outflow of a volume, r, of milk is therefore

$$t = \frac{2.3}{K} [\log 4.0 - \log (4.0 - r)] \qquad \text{(Equation 3)}$$

where r is the reading of the pipette at the point where the milk clots.

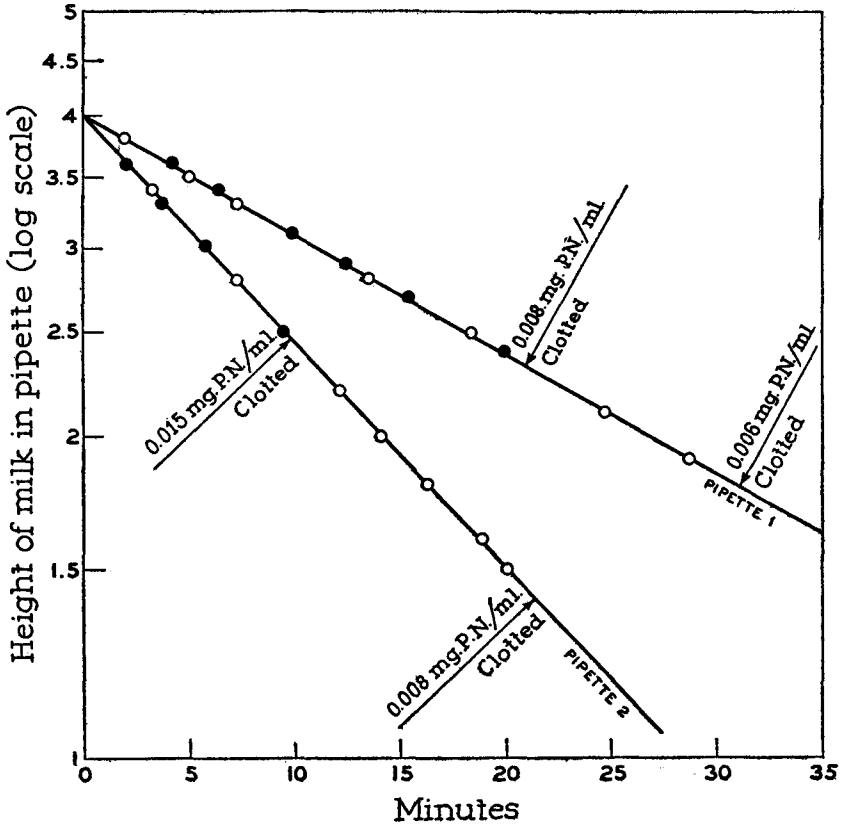

FIG. 3. Flow of 18 per cent solution of Klim milk powder containing various amounts of chymo-trypsin.

Equation 3 was checked by plotting on semi-log paper the time curves of the flow through two pipettes of milk containing various amounts of enzyme. A large number of readings were taken in this case in order to obtain many points for the curves. The curves ob-

tained (Fig. 3) are straight lines up to the very time of clotting. As the figure shows, the time of clotting of a solution of milk powder containing a definite amount of enzyme is the same for both pipettes although the rate of flow differed in the two cases.[1]

The time required for clotting of the milk is calculated as follows: Let t_1 equal the time in minutes read on the first stop-watch; *i.e.*, the time elapsed between the moment of mixing of the enzyme with the milk and the moment at which the milk passes through the 0 mark on the pipette.

t_2 equals the time read on the second stop-watch; *i.e.*, the time taken for the milk to drop from 0 to 0.5 ml.

t_3 equals the time required for the milk to drop from the zero mark to the final clotting mark, r. (The value of t_3 is calculated from Equation 3, or read off the graph, by holding a straight edge against the points (0,4) and (t_2, 3.5) on semi-log paper and then reading t_3 on the time coordinate which corresponds to the value of $4-r$ on the log coordinate). The total time required to clot the milk from the moment the enzyme is added is

$$t = t_1 + t_3$$

Definition of Rennet Unit of Activity

One unit of rennet activity $[\text{T.U.}]^{\text{Rennet}}$ is the amount of activity that causes clotting of 11 ml. of 18 per cent Klim milk in M/10 acetate buffer pH 5.0 in 1 minute at 35.5°C. The 11 ml. of mixture contains 1 ml. of enzyme solution so that if t minutes are required for the milk to clot then 1 ml. of the enzyme solution contains $\frac{1}{t}$ units of rennet activity. This last number, when divided by the milligrams protein nitrogen contained per milliliter of the enzyme solution, gives the specific activity of the enzyme per milligram protein nitrogen; *i.e.*,

$$[\text{T. U.}]^{\text{Rennet}}_{\text{mg. protein nitrogen}} = \frac{1}{t \times \text{mg. protein nitrogen/ml.}}$$

Example.—1 ml. chymo-trypsin solution in water containing 0.015 mg. protein nitrogen was added to 10 ml. 20 per cent milk solution at

[1] Fig. 3 also shows that the time required to clot is inversely proportional to the enzyme concentration, within the experimental error of about 5 per cent.

35.5° C. It required 1.6 minutes to draw the milk into the pipette, place the pipette in position, and for the milk to pass the zero mark. The time consumed in this way was read from the first stop-watch. Thus, $t_1 = 1.6$ minutes. It required 2.7 minutes for the milk to flow from 0 to the 0.5 mark as read off the second stop-watch; *i.e.*, $t_2 = 2.7$ minutes. The milk clotted when it reached the mark $r = 1.55$. The corresponding value of h_t is $4.0 - 1.55 = 2.45$. A straight line was drawn on semi-log paper through the point $t = 0$, $h = 4.0$, and the point $t = 2.7$, $h = 3.5$. The time ordinate of the point on

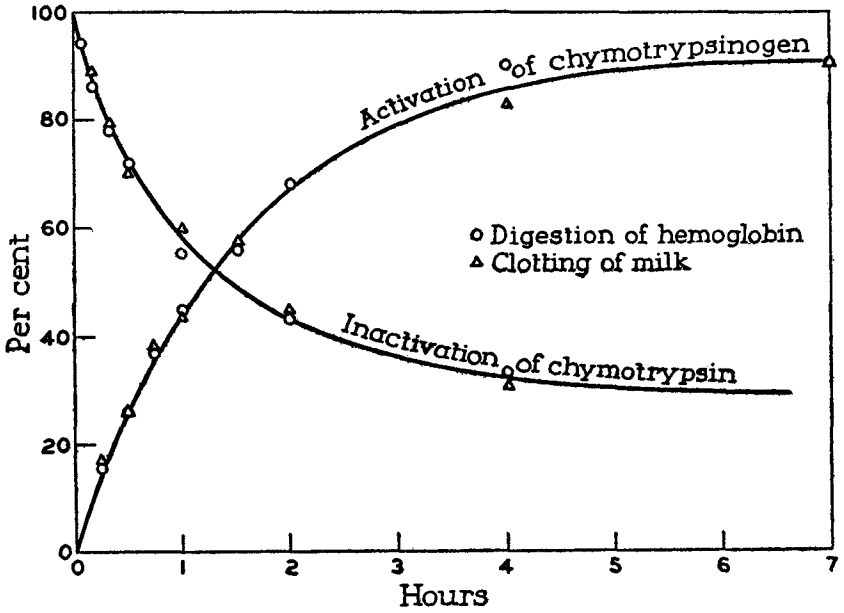

FIG. 4. Activity of chymo-trypsin as determined by its effect on digestion of hemoglobin and on clotting of milk.

this line corresponding to the value of $h = 2.45$ was $t_3 = 9.8$ minutes which equals the time required for the milk to drop from the zero mark to the clotting mark, r. The total time required to clot is

$$t = t_1 + t_3 = 11.4 \text{ minutes}$$

$$[\text{T. U.}]^{\text{Rennet}}_{\text{mg. protein nitrogen}} = \frac{1}{11.4 \times 0.015} = 5.84$$

Numerous measurements of rennet activity of chymo-trypsin have been made in connection with the experiments described elsewhere

(3). In all cases the precision obtained for the rennet activity of the chymo-trypsin by the method described here was of the same order of magnitude as that obtained in the measurements of the proteolytic activity of the enzyme. Fig. 4 shows a curve for the rate of activation of chymo-trypsinogen by trypsin, as measured by digestion of hemoglobin and by clotting of milk, expressed as per cent of final activity. The curve shows that the two methods of determining the amount of active chymo-trypsin produced at various times during activation give practically identical values. The same result is shown by the curve for inactivation of chymo-trypsin in M/10 hydrochloric acid at 25°C.

The method was found to be applicable also to the determination of the clotting of milk by pepsin. The change in viscosity preceding clotting does not cause any significant change in the rate of flow, owing to the very large aperture of the pipette.

SUMMARY

The rennet activity of chymo-trypsin (or pepsin) is conveniently measured by allowing a standard solution of milk to which chymo-trypsin has been added to flow slowly through a graduated pipette and observing the rate and distance of flow of the milk before it clots. The time required for chymo-trypsin to clot milk may be calculated from these observations. The rennet activity is expressed as the reciprocal of the time in minutes required for 1 ml. of enzyme solution to clot 10 ml. of standard milk powder solution.

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