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KINETICS OF PEPSIN-INITIATED COAGULATION OF κ-CASEIN

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Summary

The kinetics of pepsin initiated coagulation of κ-casein have been studied at pH 5.8. The primary and secondary phases of coagulation are shown to proceed simultaneously. The theory of enzymatically initiated clotting reactions proposed by Payens (Payens, T.A.J. (1976) Neth. Milk Dairy J. 30, 55—59) has been applied to this clotting system and has been used to obtain rate constants for the secondary phase of coagulation. As expected, clotting rate constants for κ-casein increase with pepsin concentration. An activation energy of 30.6 kcal/mol has been obtained for the secondary phase of coagulation. Turbidity measurements are a convenient means for studying the secondary phase of coagulation but do not provide an unambiguous means for studying the primary phase of the reaction.

Introduction

The occurrence of a lag time in the enzymatically initiated aggregation of milk suggests that the total aggregation reaction may consist of two phases. It is known that the initial phase of the reaction involves the enzymatic hydrolysis of κ-casein into para-κ-casein and a peptide called the macropeptide [1]. The secondary phase of milk coagulation involves association of casein micelles containing κ-casein into large aggregates [2]. Para-κ-casein coagulates in the absence of casein micelles and κ-casein provides, therefore, a convenient clotting substrate for studying enzymatically initiated coagulation reactions.

The lag time, commonly referred to as the clotting time, has been used traditionally as the basis for clotting enzyme assays. The Segelke-Storch relationship [3,4] is an empirically derived equation which states that the product of the clotting time and the concentration of clotting enzyme is always constant. The coagulation phase of the total clotting reaction does not appear to be an important determinant of the clotting time in this equation. This would be the case if the coagulation phase is independent of enzyme concentration. However, the

rate constant for coagulation should be dependent on enzyme concentration since the rate of coagulation must be dependent on the product of the enzymatic reaction [5,6]. The appropriateness of the Segelke-Storch relationship to describe milk coagulation can therefore be challenged. A relationship which includes terms for both the enzymatic and coagulation phases of the clotting process has been derived by Payens [7,8]. The purpose of the present study is to examine the utility of this relationship in describing the pepsin-initiated aggregation of κ-casein.

Materials and Methods

κ-Casein was purified from skim milk by the method of Zittle and Custer [9]. Discontinuous polyacrylamide gel electrophoresis [10] in the presence of 8 M urea (pH 8.7) revealed a trace (<5%) contamination with α_s-casein. Before use, freeze-dried κ-casein was disssolved in 0.1 M MES (*N*-morpholino)ethanesulfonic acid, pH 5.8. Porcine pepsin (Sigma Chemical Co., lot 67C-8195) was also dissolved in 0.1 M MES (pH 5.8) before use. Stock solutions were kept refrigerated and clotting strength of enzyme solution was tested before and after each set of experiments to insure that no deterioration of the enzyme had taken place.

The enzymatic phase of coagulation was monitored by measuring the amount of Lowry reactive material which was soluble in 12% trichloroacetic acid. Following addition of 1 ml 0.04 mg/ml pepsin to 24 ml 6.0 mg/ml substrate in 0.1 M MES (pH 5.8), 1.0 ml aliquots of this clotting solution were taken after different time intervals and added to 0.5 ml 36% trichloroacetic acid and mixed. Following centrifugation at $900 \times g$ for 15 min, the 12% trichloroacetic acid soluble peptides were analyzed by the method of Lowry et al. [11]. Color change before addition of enzyme was used as a blank. A straight-line relationship between absorbance and amount 12% trichloroacetic acid soluble peptides was obtained in the range needed for peptide-release experiments. The Michaelis-Menten constant (K_m) for the action of rennin on κ-casein is slightly less than 2 mg/ml [12,13]. Assuming a similar value when pepsin is used as enzyme, the peptide release experiments were conducted at a concentration of substrate about three times greater than K_m.

When clotting times were determined simultaneously with determination of the rate of peptide release, 0.5 ml of the above solution was transferred to a cuvette and monitored at 550 nm in a Beckman 25 spectrophotometer. In all other cases the clotting process was initiated by adding 0.4 ml substrate to 0.1 ml enzyme directly in a cuvette. Clotting times were measured by linear extrapolation of the steep portion of the plot of absorbance against time [14].

Results and Discussion

It is generally agreed that the clotting of milk consists of a primary phase (Eqn. 1) and a secondary phase (Eqn. 2). The appropriate kinetic scheme should be

$$E + S \rightleftharpoons ES \overset{k_3}{\rightarrow} E + P_1 + M \qquad (1)$$

$$iP_1 \xrightarrow{k_s} P_i \ (i = 2, 3, \ldots) \qquad (2)$$

where E refers to enzyme, S to substrate, P_1 to para-κ-casein, M to macropeptide, k_3 to the catalytic rate constant in Michaelis-Menten theory and k_s to the clotting rate constant. Although there are two major phases of this reaction, it has been difficult to separate these phases kinetically.

Fig. 1A clearly shows that the two phases of pepsin initiated coagulation of κ-casein proceed simultaneously under our experimental conditions. Coagulation, as measured by increased turbidity, begins immediately after addition of pepsin, not after completion of the enzymatic phase. The rate of release of 12% trichloroacetic acid soluble peptides is linear to times well beyond the clotting time. Thus, under these conditions the substrate does not appear to be seriously depleted during the coagulation reaction. The use of 12% trichloroacetic acid in these experiments probably results in an underestimate of the initial rate of peptide release since only glycosylated peptides are soluble [1]. However, the percent of total peptide release completed at any given time is not likely to be changed by using a lower trichloroacetic acid concentration [15]. Clearly, separation of the two phases of the reaction under these conditions is not possible. Similar difficulties in the separation of phases are present in the aggregation of fibrin [16] which, like the aggregation of milk, is an enzymatically initiated reaction [17].

Fig. 1B shows that, at a lower temperature and therefore longer coagulation time, the phases of pepsin initiated aggregation of κ-casein are more distinct kinetically. A higher percent of the total peptide release is complete by the lag time. The aggregation phase of the reaction must therefore have an activation

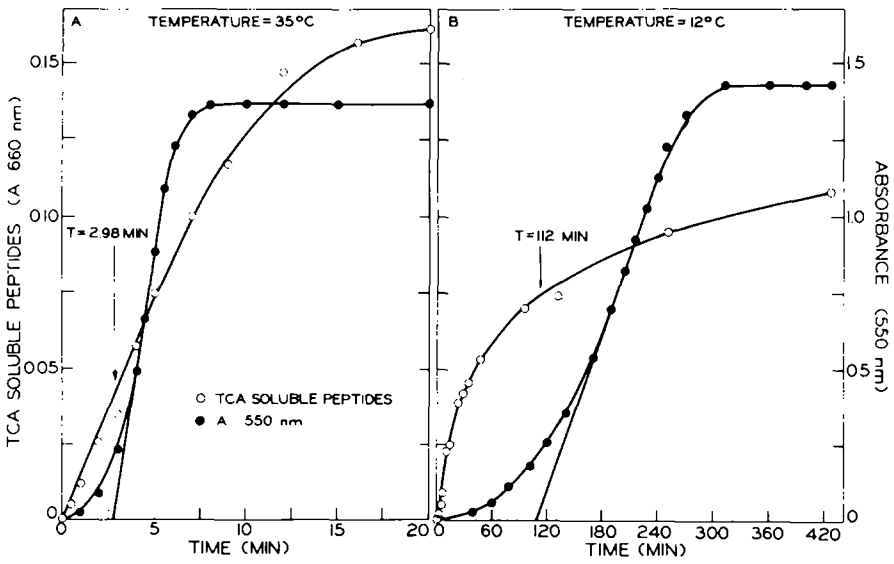

Fig. 1. Pepsin initiated coagulation of κ-casein at 35°C and 12°C. Lowry positive material soluble in 12% trichloroacetic acid and turbidity (A_{550}) are plotted as functions of time, after addition of enzyme. (1.6×10^{-3} mg/ml pepsin, 5.8 mg/ml κ-casein, 0.1 M MES (pH 5.8)).

energy substantially higher than does the enzymatic phase. Similar temperature-dependent behavior has been observed in the coagulation of whole milk [18]. Although the phases appear to be at least partially separated at this temperature, it is not possible to study them in isolation. This is in contrast to the coagulation of whole milk, which will not clot at low temperature [19].

One approach towards separation of the enzymatic and coagulation phases of this reaction has been proposed by Payens [7,8] who assumes that the enzymatic phase follows Michaelis-Menten enzyme kinetics and that the coagulation phase follows von Smoluchowski's theory of coagulation kinetics [5,6]. Using this approach, Payens has argued that the appropriate relationship for the enzymatic clotting time is

$$T(k_s v/2)^{1/2} = C \tag{3}$$

where T is the observed clotting time, k_s is the second order clotting rate constant from von Smoluchowski's theory, v is the rate of the enzymatic phase of coagulation, and C is a constant. This equation has two clear advantages compared to the Segelke-Storch relationship. First, it states explicitly what is obvious from Fig. 1, that is, clotting times are dependent on coagulation rates as well as enzymatic velocities. And second, it rests on a believable theoretical foundation rather than being an empirical equation.

Using assumptions similar to those required to derive Eqn. 3, Payens [8,20] has derived a theoretical relationship between weight-average molecular weight ($\overline{M}_w$) and time (t), during an enzyme initiated clotting reaction. When the effect of the initial enzymatic hydrolysis reaction on weight-average molecular weight is a negligible part of the overall change during clotting, it can be shown that:

$$\overline{M}_w = M_o + (M_o^2 C^3/3c_o)(8v/k_s)^{1/2}(t/T)^3 \tag{4}$$

where M_o is molecular weight of the clotting substrate and c_o is initial concentration of the substrate in units of g/ml. By combining Eqn. 3 with Eqn. 4, the following is obtained:

$$\overline{M}_w = M_o + (4M_o^2 C^4/3c_o k_s T^4)t^3 \tag{5}$$

This is an equation for a straight line where the clotting rate constant may be calculated from the slope. If weight-average molecular weight is approximately proportional to turbidity, it should be possible to obtain the clotting rate constant from the relationship

$$m = 4M_o^2 C^4/3c_o k_s T^4 \tag{6}$$

where m is the slope of the plot of absorbance versus time raised to the third power. The clotting rate constant could then be obtained under a variety of experimental conditions.

Payens [20] was unable to obtain values for m using a casein-micellar substrate, a fact which he attributed to multiple scattering effects in the micellar system. The use of a lower molecular weight substrate, κ-casein, reduces these effects, and plots of absorbance versus time to the third power yield straight lines until well beyond clotting times (Fig. 2). Since the slopes of these plots increase with enzyme concentration, the clotting rate constant is dependent on

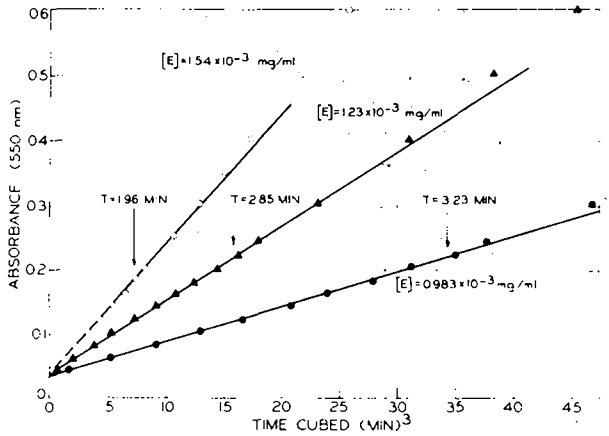

Fig. 2. Turbidity changes (A_{550}) after addition of pepsin to κ-casein plotted as a function of time raised to the third power (min^3). (0.72 mg/ml κ-casein, 0.1 M MES (pH 5.8), 35°C).

enzyme concentration. As shown in Fig. 3, relative values for the clotting rate constant increase as enzyme concentration increases. Eqn. 3 predicts that a plot of log T against $\log(k_s E)^{-1}$ will be a straight line with a slope equal to 0.5. The experimental value for this slope actually obtained was 0.475 with a correlation coefficient of 0.9989. It can be concluded that if k_s is considered as a variable along with T and v, Eqn. 3 correctly describes the clotting reaction. It should be noted that the substrate concentration used in these experiments (0.72 mg/ml) is probably less than K_m for the enzymatic reaction. In spite of this, the slopes of lines shown in Fig. 2 appear to be constant to times well past the lag time suggesting that calculated values of k_s are not dramatically affected by substrate depletion under these conditions.

It should be noted that the Segelke-Storch relationship predicts that a plot

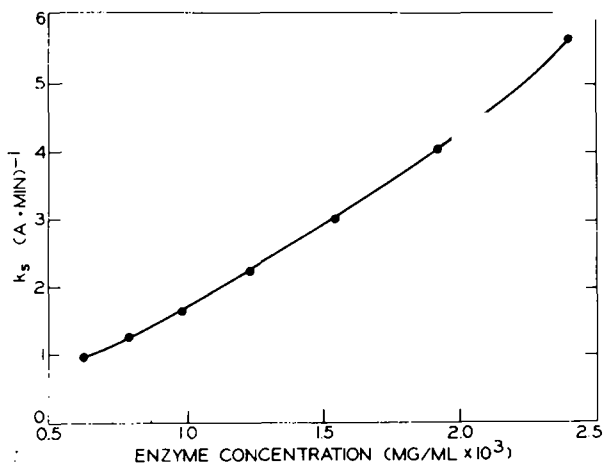

Fig. 3. Effect of pepsin concentration on clotting rate constant. Clotting rate constant was calculated from Eqn. 6. (0.72 mg/ml κ-casein, 0.1 M MES (pH 5.8), 35°C).

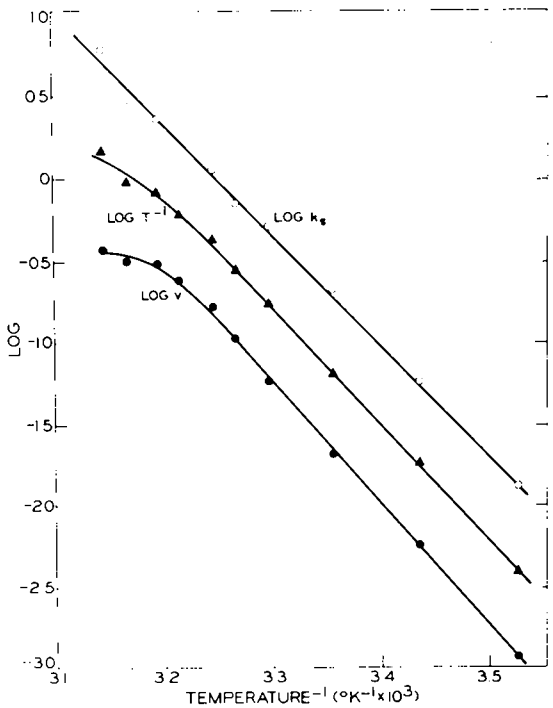

Fig. 4. Arrhenius plots for clotting rate constant (k_s), reciprocal clotting time (T^{-1}) and velocity of enzymatic phase of coagulation (v). k_s was calculated from Eqn. 6, v was calculated from Eqn. 3. (2 × 10^{-3} mg/ml pepsin, 5.7 mg/ml κ-casein, 0.1 M MES (pH 5.8)).

of log T against log $[E]^{-1}$ will be a straight line with a slope of 1.0. Our data agree quite well with this expectation also, giving an experimental slope of 1.10 with a correlation coefficient of 0.9990. Both the Segelke-Storch relationship and Eqn. 3, therefore, adequately describe the dependence of clotting time on enzyme concentration. It would seem, however, that the practical utility of the Segelke-Storch equation derives from the fact that the clotting rate constant is linearly dependent on enzyme concentration (see Fig. 3). Notice that Eqn. 3 reduces to the Segelke-Storch relationship when both k_s and v are linearly related to [E]. In addition to putting the clotting reaction on a reasonable theoretical basis, we shall see that Eqn. 3 provides useful insights into the temperature dependence of the clotting reaction.

Arrhenius plots for aggregation of κ-casein are curved when reciprocal clotting times are used as the measure of the rate of the clotting reaction [21]. It has been suggested on this basis that different steps of the clotting reaction are rate limiting at different temperatures [21]. The middle curve in Fig. 4 shows that the Arrhenius plot for reciprocal clotting time under the present experimental conditions is also curvilinear, especially at higher temperatures when clotting times are short. The upper curve in Fig. 4 shows that an Arrhenius plot for k_s values obtained as described above is linear over the entire temperature range with an activation energy of 30.6 kcal/mol. The lower curve in Fig. 4 is an Arrhenius plot for values of v calculated by inserting measured

values for T and k_s into Eqn. 3. The plot is, in this instance, even more curvilinear than the plot for reciprocal clotting times. An activation energy of 10.2 kcal/mol is obtained for v using the four highest temperatures. This is in the range expected for enzymatic hydrolysis of κ-casein [2,12,22,23] and in fair agreement with the activation energy for peptide release calculated from the two experiments shown in Fig. 1 (14.1 kcal/mol). The alteration in the slope of the Arrhenius plot for v at lower temperatures suggests that k_s has become rate limiting under these conditions. Eqns. 3 and 4 appear to more accurately describe the progress of the clotting reaction at higher temperatures when clotting times are short than at lower temperatures when clotting times are long. The linearity of the Arrhenius plot for k_s over the entire temperature range studied (45.0—10.5°C) seems to us to be a clear indication of the utility of these equations and support Payens observation that turbidity measurements provide an adequate means for obtaining the clotting trate constant. It also seems to indicate that they provide a means of obtaining values for k_s even when depletion of substrate begins well before the clotting time (Fig. 1B). Turbidity measurements do not appear, however, to be an unambiguous means for determining the rate of the primary phase of the total reaction under all conditions.

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