Activation Studies of the Multiple Forms of Prochymosin (Prorennin)

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Activation of the four separate components of prochymosin (prorennin) at pH 5.0 demonstrated that each zymogen was the precursor to an electrophoretically distinct chymosin (rennin). When the increase in milk-clotting activity with time was analysed, the mechanism of activation of unfractionated prochymosin, individual prochymosin components, and a mixture of the prochymosin fractions at pH 5.0 was shown to follow essentially autocatalytic kinetics. The activation of prochymosin C was completed in 70h, whereas the other three fractions each required more than 110h for complete activation under the same conditions. Intact prochymosin, the mixture of four components and prochymosin C were activated at similar rates. Interaction of the individual fractions during activation is suggested to explain the increased rate of the activation for the mixture. Comparison of autocatalytic activation of unfractionated prochymosin purified chromatographically at pH 6.7 and 5.7 demonstrated an increased rate of reaction for the zymogen prepared at the lower pH value. The possibility that prochymosin became susceptible to activation during preparation at pH values slightly below 6.0, as a result of changes in the proportion of the components or a conformational change and exposure of the active site, is discussed.

The precursors of proteolytic digestive enzymes, termed zymogens, are released to the surface of the particular organ where they are activated and function. Prochymosin (prorennin), the zymogen of the milk-clotting enzyme chymosin (rennin) (EC 3.4.23.4), is found in the abomasum (fourth stomach) of young milk-fed calves. The conversion of prochymosin into chymosin involves the hydrolysis of peptides from the *N*-terminal region of prochymosin (Foltmann, 1964).

Foltmann (1962, 1966) demonstrated that activation of two chromatographic fractions of prochymosin resulted in the formation of chromatographically different chymosin components. Crystalline chymosin was resolved into three chromatographic fractions, and Foltmann (1962, 1966) discussed the possible existence of three separate zymogens to account for the origin of the chymosin peaks. Bundy *et al.* (1967) studied the activation of a homogeneous prochymosin preparation at pH 3.2 by paper electrophoresis and found that only one chymosin component was formed. Shukuri (1970) also prepared prochymosin consisting of a single component, when assayed by chromatography on DEAE-cellulose at pH 5.8 and electrophoresis in starch/urea gel at pH 8.3. The chymosin obtained from this prochymosin by activation at pH 2.0 or 5.0 was reported

to be essentially homogeneous. However, crystalline chymosin was resolved into six components by the starch/urea-gel-electrophoretic technique. Shukuri (1970) discussed the possibility that the several chymosin components, distinguishable by starch/urea-gel electrophoresis, were partially fragmented forms of a chymosin. He also found differences between chymosin formed by activation at pH 2.0 and 5.0 when characterized by the behaviour on DEAE-cellulose column chromatography, electrophoresis and amino acid analysis. Asato & Rand (1971) have established that there are multiple forms of both prochymosin and chymosin which can be distinguished by polyacrylamide-gel electrophoresis. Asato & Rand (1972) fractionated prochymosin into four components by chromatography on DEAE-cellulose and have shown that the individual prochymosin components have distinctly different mobilities in polyacrylamide-gel electrophoresis. Chymosin was also shown to consist of four components by electrophoresis and the existence of one zymogen for one enzyme was indicated.

Rand & Ernstrom (1964) investigated the activation of prochymosin between pH 2.0 and 5.5. They demonstrated that the rate of activation of prochymosin increased with decreasing pH below 5.5 and that the kinetics at pH 4.7 and 5.0 were predominantly autocatalytic. The rate of prochymosin activation continued to increase below pH 4.0, although past the region of optimum proteolytic activity for chymo-

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sin, and became too rapid, even at low temperatures, to follow accurately.

The purpose of the present study was to examine the autocatalytic activation of whole prochymosin and the individual prochymosin components. The reaction was carried out at pH 5.0 and 25°C, since this was compatible with both autocatalysis and chymosin stability (Rand & Ernstrom, 1968; Mickelsen & Ernstrom, 1967). This approach was used to establish whether each component of prochymosin was converted into a separate fraction of chymosin by an autocatalytic mechanism. It was also possible to investigate the interaction between zymogen components and their enzymes during autocatalysis.

Experimental

Materials

Non-fat dry milk was used for the substrate and dry salted calf stomachs were the source of prochymosin (Asato & Rand, 1972). Crystalline chymosin was prepared by the method of Ernstrom (1958).

Methods

Enzyme activity. The chymosin unit and the determination of prochymosin activity as a potential chymosin unit have been previously defined on the basis of milk-clotting activity (Rand & Ernstrom, 1964; Asato & Rand, 1972).

Purification of prochymosin. Prochymosin was extracted from dry salted calf stomachs and the insoluble material was removed by centrifugation (Asato & Rand, 1971). The supernatant was dialysed against several changes of 0.2M-disodium phosphate solution (pH 7.5). The crude prochymosin extract was purified at pH 5.7 by chromatography on DEAE-cellulose by method (2) as reported by Asato & Rand (1972). A chromatographic purification of prochymosin was also carried out at a higher pH value to investigate the possibility of activation changes occurring in prochymosin purification at pH values below pH 6.0. Freeze-dried crude prochymosin extract was redissolved in 0.01M-sodium phosphate buffer, pH 7.0, and applied to a DEAE-cellulose column equilibrated with 0.05M-sodium phosphate buffer (pH 6.9). Elution was carried out by a stepwise procedure, with 500 ml of 0.05M-sodium phosphate buffer, pH 6.9, 500 ml of 0.1M-sodium phosphate buffer, pH 6.8, and 900 ml of 0.2M-sodium phosphate buffer, pH 6.7. All prochymosin fractions with activity were pooled, dialysed against 0.2M-disodium phosphate solution, pH 7.5, and freeze-dried.

Fractionation of prochymosin. The chromatographic resolution of prochymosin into four components was performed by method (2) as described by Asato & Rand (1972).

Activation of prochymosin. Activation of prochymosin was carried out in 0.3M-sodium citrate buffer at pH 5.0. A freeze-dried prochymosin preparation (20mg) was dissolved in 1 ml of water and activated by the addition of 1 ml of 0.6M-sodium citrate buffer, pH 5.0, at 25°C in a water bath. Samples (0.1 ml) were withdrawn from the activation mixture and added to 1 ml of water. After appropriate dilution with water, a 1 ml portion was assayed for milk-clotting activity. The mixture of four prochymosin components was prepared by mixing 4mg of component D (fraction I), 6mg of component C (fraction II), 8mg of component B (fraction III) and 2mg of component A (fraction IV), giving the proportions 2:3:4:1. This value was estimated from the protein concentration required for the same relative intensity of each prochymosin band when stained with Coomassie Brilliant Blue R250 dye after electrophoresis on polyacrylamide gel. Activation of prochymosin at pH 5.0 for electrophoretic analysis was carried out as previously described (Asato & Rand, 1971).

Polyacrylamide-gel electrophoresis. Electrophoresis of prochymosin and chymosin was conducted with 6.5% (w/v) polyacrylamide gels containing 0.1% casein at pH 7.1 in a vertical cell by the procedure of Asato & Rand (1971).

Results

Activation of prochymosin

The activation of intact prochymosin purified chromatographically at pH 5.7 and at 6.7 is compared in Fig. 1. The prochymosin purified at the lower pH was completely converted into chymosin in 70 h, whereas conversion of the prochymosin purified at

Fig. 1. Activation at pH 5.0 and 25°C of prochymosin purified at pH 5.7 (○) compared with prochymosin purified at pH 6.7 (△)

The enzyme activity formed at each time interval was measured as milk-clotting activity and expressed as chymosin units.

Table 1. Comparison of the time required for 50% activation and the activity at zero time for intact and individual prochymosin preparations

The time required for 50% activation, or the maximum rate, was obtained from Figs. 1 and 3. Activity at zero time is expressed in chymosin units (see the Experimental section).

Zymogen preparation	Time required for 50% activation (h)	Activity at zero time (chymosin unit/ml)
Fraction I (prochymosin D)	50	0.11
Fraction II (prochymosin C)	27	0.22
Fraction III (prochymosin B)	54	0.23
Fraction IV (prochymosin A)	57	0.17
Prochymosin mixture	30	0.70
Prochymosin prepared at pH 5.7	38	0.14
Prochymosin prepared at pH 6.7	56	0.13

Fig. 2. Plots of $\ln[A/(A_e - A)]$ versus time for activation at pH 5.0 of prochymosin purified at pH 5.7 (O) and at pH 6.7 (Δ)

The term A is the chymosin activity formed at any given time of activation and A_e is the final or equilibrium activity.

pH 6.7 required 130h. This result indicated that exposure of prochymosin to a pH slightly below 6.0 during purification accelerated the activation reaction. The difference in rate of activation might be explained by a difference in the ratio of preformed chymosin to prochymosin, which was slightly higher for prochymosin purified at pH 5.7 than for prochymosin purified at pH 6.7. However, the difference in the amount of preformed chymosin between the two prochymosin preparations, shown in Table 1, ap-

pears to be too small to account for this difference. It was assumed that prochymosin became susceptible to activation because of a conformational change, which took place as a result of the exposure of the zymogen during preparation to a pH slightly below

Fig. 3. Activation of fractionated prochymosin components and a formulated mixture of the prochymosin components when incubated at pH 5.0 and 25°C

The enzyme activity formed at each time interval was measured as milk-clotting activity and expressed as chymosin units. O, Prochymosin A; ●, prochymosin B; Δ, prochymosin C; ▲, prochymosin D; □, prochymosin mixture.

Fig. 4. Plots of $\ln[A/(A_e - A)]$ versus time for activation at pH 5.0 of the fractional prochymosin components and a formulated prochymosin mixture

The term A is the chymosin activity formed at any given time and A_e is the final activity. O, Prochymosin A; ●, prochymosin B; Δ, prochymosin C; ▲, prochymosin D; □, prochymosin mixture.

Fig. 5. Application of electrophoresis to follow the course of prochymosin activation at pH 5.0

The negative pole was at the top of the gel where the sample numbers are located, with migration toward the positive pole at the bottom. Activation samples: (1) 0h; (2) 10h; (3) 20h; (4) 30h; (5) 40h; (6) 50h; (7) 60h; (8) 70h; (9) 80h; (10) crystalline chymosin. (b) is a schematic representation of (a).

6.0. At pH 5.0, the activation reaction appears to be autocatalytic, as indicated by the typical S-shaped curves. This is confirmed by kinetic linear plots according to Kunitz & Northrop (1936) as shown in Fig. 2.

Activation of the individual prochymosin components and a mixture was studied at pH 5.0 and the results are shown in Fig. 3. In general the activation

Fig. 6. Electrophoretic analysis of prochymosin, prochymosin components, prochymosin components activated at pH 5.0 and crystalline chymosin

The negative pole was at the top of the gel, with migration toward the positive pole at the bottom. Samples: (1) prochymosin; (2) prochymosin D; (3) prochymosin C; (4) prochymosin B; (5) prochymosin A; (6) activated prochymosin D; (7) activated prochymosin C; (8) activated prochymosin B; (9) activated prochymosin A; (10) crystalline chymosin. (b) is a schematic representation of (a).

of the prochymosin fractions and the mixture of these fractions followed a sigmoidal course, which is substantiated by the kinetic plots shown in Fig. 4. Prochymosin C was activated rapidly and the re-

action was essentially complete in 70h, whereas activation of the other three components was slow and required at least 110h for maximum chymosin formation. The activation rate for the mixture of prochymosin components was almost the same as for prochymosin C and intact prochymosin prepared at pH 5.7 as shown in Fig. 1. These comparisons in the rate of activation can be clearly demonstrated with the time required for 50% activation, which is presented in Table 1 together with the initial chymosin activity at zero time.

Electrophoresis of activated prochymosin and prochymosin components

Activation of intact prochymosin purified at pH 5.7 was carried out at 25°C in the 0.015M-imidazole/citrate buffer, pH 5.0, of Asato & Rand (1971). Samples were withdrawn at 10h intervals from 0 to 80h and neutralized to pH 6.3. The activation samples of prochymosin were analysed simultaneously along with crystalline chymosin in a polyacrylamide gel as shown in Fig. 5. With increasing activation time, the potential proteolytic activity of the four prochymosin bands of intact prochymosin decreased, whereas the activity of the four individual chymosin components increased at almost equal rates. This result indicated that, as activation proceeded, four zymogen components gave rise to four separate enzymes.

To confirm the concept that each zymogen gives rise to a separate enzyme, the individual prochymosin components were activated at pH 5.0 and analysed electrophoretically along with crystalline chymosin. The conversion of individual prochymosin components into chymosin was accomplished by incubation at 25°C in a water bath over a period of 80h for prochymosin C, 110h for prochymosin B and 130h for prochymosins D and A. The result of electrophoretic analysis of DEAE-cellulose-purified prochymosin zymogen components A, B, C and D, the activated prochymosin components A, B, C and D, and crystalline chymosin is shown in Fig. 6. This demonstrates that activation of the individual prochymosin components at pH 5.0 gave rise to correspondingly separate chymosins. The order of mobility for the activated prochymosin components was the same as that of the zymogen components.

Discussion

Activation of DEAE-cellulose-purified prochymosin containing four components at pH 5.0 resulted in the formation of four different chymosins. It was also demonstrated that each prochymosin component was a precursor of an individual enzyme, as predicted previously (Asato & Rand, 1972). This was contrary to the reports of Bundy *et al.* (1967) and Shukuri (1970), but partly agrees with the findings of

Foltmann (1962, 1966). In electrophoresis, all four enzymes moved ahead of the zymogens to the cathode; thus the conversion from prochymosin into the active enzyme involves the release of peptides with a higher proportion of basic amino acids. The active enzymes all maintained the same relative mobility and position in gel electrophoresis as those of the four prochymosins, indicating that the peptide(s) released from all zymogens were similar. The separation of zymogens and enzymes is attributed to the difference in negative charge between precursors and active enzymes.

The activation of prochymosin was studied by following the increase in milk-clotting activity with time at pH 5.0. In autocatalytic activation (Kunitz & Northrop, 1936), the set would be:

$$[A > 0, A_e > 0, K > 0: F(A) = KA(A_e - A)]$$

where A is activity at time t , A_e final or equilibrium activity, K the autocatalytic constant and $F(A)$ a function of A .

The maximum rate of formation of A was found with the standard technique:

$$F(A) = \frac{dA}{dt} = KA(A_e - A)$$

$$F'(A) = K(A_e - 2A)$$

Therefore, when $F'(A) = 0$, $A = \frac{1}{2}A_e$, $F''(A) = -2K$, and, since $K > 0$, this implies that $F''(A) < 0$, and hence $A = \frac{1}{2}A_e$ is the point of the maximum rate of formation of A with respect to activity. The time required for 50% activation, where the maximum rate of activation takes place, is obtained from the activation curve and used for characterization of the activation reaction of zymogens when activity at zero time (A_0) is known.

To investigate the origin of activity at zero time ($A_0 > 0$), the activation of prochymosin purified at pH 5.7 was compared with that of prochymosin purified at pH 6.7. The former was activated faster than the latter, as indicated by the time required for 50% activation. This difference in the activation rate could be attributed to alterations in the ratio of the prochymosin and chymosin components caused by the purification procedures. However, electrophoretic analysis of the two preparations did not reveal any obvious changes in the protein concentration or enzyme activity within the four component systems. The increased rate of autocatalysis was apparently caused by the exposure of prochymosin to a pH below 6.0. Foltmann (1966) suggested that prochymosin could be gradually activated by a conformational change at pH values slightly below 5.0. He assumed that this conformational change in the prochymosin molecule went from 'closed' to 'open' and uncovered the active site and accelerated the conversion of prochymosin into chymosin. This conformational

change would be sufficient to initiate the activation of prochymosin. This view, that a zymogen can catalyze its own activation, was further confirmed by the incubation of a polyacrylamide gel containing casein in a buffer of pH 5.9 after separation of any chymosin from the main prochymosin band by electrophoresis. Incubation of the gel in 0.3M-sodium citrate buffer, pH 5.9, at 25°C for 40h demonstrated the activation of prochymosin by digestion of the substrate *in situ*. Bustin & Conway-Jacobs (1971) demonstrated that the activation of pig pepsinogen at pH 3.0 and 23°C could be an intramolecular reaction when pepsin activity was inhibited by an excess of haemoglobin substrate, and indicated that protonation of the proenzyme initiated the conformational change and exposed the active site. This result is compatible with Perlmann's (1967) conclusion that electrostatic interaction between the basic amino acid residues of the peptides released on activation and carboxylic acids of the pepsin moiety contributed to the stabilization of the configuration of pepsinogen at a pH above 6.0. Funatsu *et al.* (1971, 1972) proposed a similar mechanism from studies of pepsinogen activation in the presence of inhibitors, 3-methylbutan-1-ol and *N*-benzyloxycarbonylglut-
amylphenylalanine.

Kassell & Kay (1973) have reviewed the status of this concept that zymogens possess inherent activity as first proposed by Foltmann (1966). Under appropriate conditions, enzymic activity has now been demonstrated for a variety of the proteolytic zymogens. In addition, it seems certain that the autocatalytic zymogens, pepsinogen and trypsinogen, can be activated in monomolecular reactions without a trace of active enzyme. Prochymosin appears to have a similar capability.

The autocatalytic activation rate for the individual prochymosin components falls into essentially two classes. Prochymosin C was activated rapidly, but the conversion of prochymosins D, B and A into the corresponding chymosin took nearly twice as long. However, the mixture of these prochymosin fractions was activated rapidly at a rate about equal to that for prochymosin C and intact prochymosin (Fig. 1). This suggests that a non-specific activation of the individual prochymosin components occurs, with chymosin formed from prochymosin C activating not only prochymosin C, but also prochymosins D, B and A. To explain the higher rate of activation for the prochymosin-fraction mixture, it is possible to assume that interaction occurs between the individual zymogen and enzyme components during autocatalysis. Mitochondrial monoamine oxidase from different areas of the human brain has also been shown to consist of four bands (MAO₁₋₄) on polyacrylamide-gel electrophoresis (Collins *et al.*, 1970). Component MAO₁ had the highest specific activity towards

tryptamine, tyramine and benzylamine. The results also indicated that the specific activity of the monoamine oxidase complex was greater than that expected from individual enzyme components towards tryptamine, tyramine and benzylamine. Collins *et al.* (1970) concluded that the multiple forms of monoamine oxidase are conformational isoenzymes. However, it is more likely that the multiple forms of prochymosin are homologous in nature, as for proteolytic enzymes (Neurath *et al.*, 1967). It appears that when the multiple forms of prochymosin are brought together, these molecules became susceptible to activation under autocatalytic conditions by a complementary effect.

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