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Prochymosin activation by non-aspartic proteinases

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Prochymosin can be converted into chymosin by an action of external proteinases. Thus, thermolysin at pH 5.05 converts calf prochymosin into active Phe-chymosin, which is one amino acid longer than chymosin from the N-terminus with a yield of 73%. Even better results were achieved with prochymosin activation by *Legionella pneumophila* metalloproteinase. Apparently the stretch of prochymosin polypeptide chain adjacent to the normally observed activation point becomes available for an attack by an external proteinase at pH 5.0–6.0. These data indicate that the intermolecular activation pathway might be of physiological importance.

Chymosin; Prochymosin activation; Metalloproteinase; Thermolysin; Phe-chymosin

1. INTRODUCTION

The activation of aspartic proteinase zymogens always proceeds by limited proteolysis that leads to the removal of a relatively long (ca. 40–45 residues) polypeptide stretch from the amino terminus of the zymogen [1]. It might be presumed that the main features of the prochymosin tertiary structure as well as the pattern of its activation should be similar to those of pepsinogen [2]. Two alternative pathways have been suggested for the activation of aspartic proteinase zymogens. The 'internal' one includes intramolecular self-activation as a crucial step. It is accepted that the first step consists of spatial rearrangement of the zymogen that brings one of the bonds within the propeptide sequence near to the catalytic site of the future enzyme to be cleaved there. This intramolecular reaction serves as an initiation step, and newly formed active aspartyl proteinase might further activate other zymogen molecules via intramolecular proteolysis.

An alternative ('external') scheme suggests that the activation might be initiated by an intermolecular reaction between the zymogen molecules that have acquired the conformation characteristic for an active enzyme and other zymogen molecules which are still inactive. Apparently, the zymogen molecule that serves as a substrate for the 'active' ones should be adapted for this role. Although data on the physiological pathway of pepsinogen activation are still rather scarce, it is presumed to proceed autocatalytically without the involvement of other proteolytic enzymes. Pepsinogen activation by an enzyme different from aspartic proteinases was reported earlier [3]. Plasmin and tissue plasminogen activator were shown to activate prorenin [4].

Swine pepsinogen was activated by *Aspergillus oryzae* metalloproteinase at pH 5 with the formation of almost 100% leucyl-pepsin. Metalloproteinase from *Legionella pneumophila*, thermolysin, serine proteinase from *A. oryzae* as well as pancreatic elastase revealed comparable activation efficiency [5]. We applied the same approach to study 'external' prochymosin activation by non-aspartic proteinases.

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2. MATERIALS AND METHODS

2.1. *Zymogen and enzymes*

Prochymosin was isolated from dried calf stomachs [6]. It gave one major band after SDS-gel electrophoresis and showed only marginal milk clotting activity that never exceeded 3% of those measured after its activation at pH 2. Thermolysin was purchased from Serva. Metalloproteinase from *L. pneumophila* [7], and serine proteinase from *A. oryzae* were isolated by E. Vashkevitch and T.I. Vaganova.

2.2. *Milk clotting activity*

Milk clotting activity was measured as follows. 500 mg of lyophilized defatted milk were dissolved in 100 ml 0.1 M acetate buffer, pH 5.6, to which 1 ml of 3 M $CaCl_2$ was added. To 3 ml of this mixture preincubated for 3 min at 37°C, 30 μl of the enzyme solution (25 μg/ml) were added. The specific activity was calculated according to the following formula: specific activity = $1/(A_{280} \cdot V \cdot t)$, where A_{280} = the optical density of the enzyme solution at 280 nm, V = the volume of the enzyme sample in ml, and t = the clotting time in s.

2.3. *Prochymosin spontaneous activation at pH 2*

To 200 μl of prochymosin solution (0.2 mg/ml) in 0.1 M acetate buffer, pH 6.0, 200 μl of 0.2 M NaCl solution in 0.25 M HCl was added to reach pH 2. The mixture was kept at 20°C for 90 min, then 3.6 ml of 50 mM phosphate buffer, pH 6.3, was added and the activity was measured.

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2.4. *Prochymosin activation with thermolysin*

To a solution with 10.15 mg of prochymosin in 39 ml of 0.1 M acetate buffer, pH 5.1, 0.1 mg of thermolysin was added (100:1 prochymosin-enzyme ratio). After 1 h at 37°C, the reaction was stopped by addition of 1.3 ml of 0.15 M Na_2EDTA. The mixture was applied on a DEAE-cellulose DE-32 Whatman column, equilibrated with 0.1 M acetate buffer, pH 5.6, and then the enzyme was eluted with 0.2 M acetate buffer, desalted on Sephadex G-25 and lyophilized which gave 4.6 mg of Phe-chymosin with a specific activity of 30 units. In the absence of thermolysin, only 3% of the maximal milk clotting activity appeared.

3. RESULTS AND DISCUSSION

We have shown that prochymosin in analogy to pepsinogen can be activated by various non-aspartic proteinases. Thermolysin at pH 5.05 converts prochymosin into active enzyme (fig.1). The highest yield (73%) was attained at a thermolysin:prochymosin ratio of 1:250 after 2.5 h at 37°C. The activation proceeds even faster at a 1:100 ratio, although the high concentration of thermolysin caused a slow degradation of the protein substrate. Even at a 1:1000 ratio, ca. 60% yield was observed. Under these conditions, self-activation of prochymosin appeared to be of minor importance attaining ca. 10% after 24 h. Obviously, the activation of chymosin by external metalloproteinase proceeds faster than that of pepsinogen [5]. Remarkably enough, the activation of prochymosin by thermolysin proceeds as well at pH 5.88; the yields approach 45–50% after 24 h. At pH 6.4 when no autocatalytic activation was observed, prochymosin was converted into active enzyme by thermolysin with a 20–25% yield after 24 h. The yield of activated chymosin is always limited by concurrent non-specific degradation of both prochymosin and chymosin by thermolysin. To identify the product of activation, the reaction mixture obtained after 1 h at pH 5.05 and a thermolysin:prochymosin ratio of 1:100 (apparent yield = 50%) was fractionated by DEAE-cellulose chromatography (table 1). The protein thus isolated was essentially homogeneous as judged by PAAG electrophoresis. Its N-terminal amino acid sequence determined by automatic Edman procedure Phe-Gly-Glu-Val-Ala-Ser-Val-Pro- is one amino acid (Phe) longer than that of 'conventional' chymosin.

Table 1

Isolation of Phe-chymosin produced by thermolysin-catalyzed activation of prochymosin

Operation	A_{280}	Activity		Yield (%)
		Total units	Specific units/A_{280}	
Activation mixture	14.3	177	12.4	100
DEAE-cellulose chromatography	7.4	297	35	145
Sephadex G-25 gel filtration	6.6	195	30	110

Hence, thermolysin at pH 5.05 cleaves in the prochymosin sequence the peptide bond Gly-Phe preceding the one that is hydrolyzed in the course of prochymosin 'normal' activation.

The peptide bonds cleaved by thermolysin, *Legionella* metalloproteinase and chymosin are as follows:

thermolysin ↓ ↓chymosin

Legionella metalloproteinase ↓

Ser-Lys-Tyr-Ser-Gly-Phe-Gly-Glu-Val-Ala-Ser-Val-Pro

propeptide ——→ <Phe-chymosin ——————

<chymosin ——————

This resembles the activation of swine pepsinogen by metalloproteinase from *A. oryzae* which also made the enzyme one amino acid longer than 'natural' pepsin, Leu-pepsin [5]. In both cases, thermolysin cleaves in accordance with its specificity the bond formed by the amino group of the hydrophobic amino acid. An even better result was achieved by the activation of prochymosin by a metalloproteinase secreted by *L. pneumophila*. A 70% yield of Phe-chymosin was achieved in 1 h when a 1:100 ratio of enzyme:prochymosin was used and even at a 1:1000 ratio the same yield was attained after 5 h. Phe-chymosin isolation gave an even better yield due to the removal of inhibitory admixtures. Hence, the activation extent found in the analytical experiments represents the lower limit.

A. oryzae serine proteinase activated prochymosin at pH 5.0, 5.6 and 5.88 with a maximal yield of 10, 10 and 6%, respectively, due to strong non-specific hydrolysis.

These experiments lead us to the following conclusions:

(1) It appears that the amino acid sequence of prochymosin surrounding the future amino terminus of chymosin is in a conformation favorable for its interaction with metalloproteinases as well as with certain serine proteinases. Presumably this stretch of the polypeptide chain acquires a conformation that allows several amino acid residues, located before and after the bond to be cleaved, to interact with the substrate binding site of the activating metalloproteinase. It might refer to P_1, P_2, P_3, P_1', P_2' residues. Hence, this segment of the zymogen sequence appears to be adapted to external proteinase attack even at rather high pH, when autocatalytic activation would not proceed.

The activation by metalloproteinases proceeds much more efficiently at pH 5, than at pH 5.88 or 6.4, although their intrinsic activity diminishes as the pH drops. This should be ascribed to a pH-dependent conformational change that exposes the relevant stretch of the prochymosin sequence to a proteinase.

(2) The very fact that various proteinases can activate pepsinogen and prochymosin cleavage of different, although neighboring, peptide bonds indicates that the

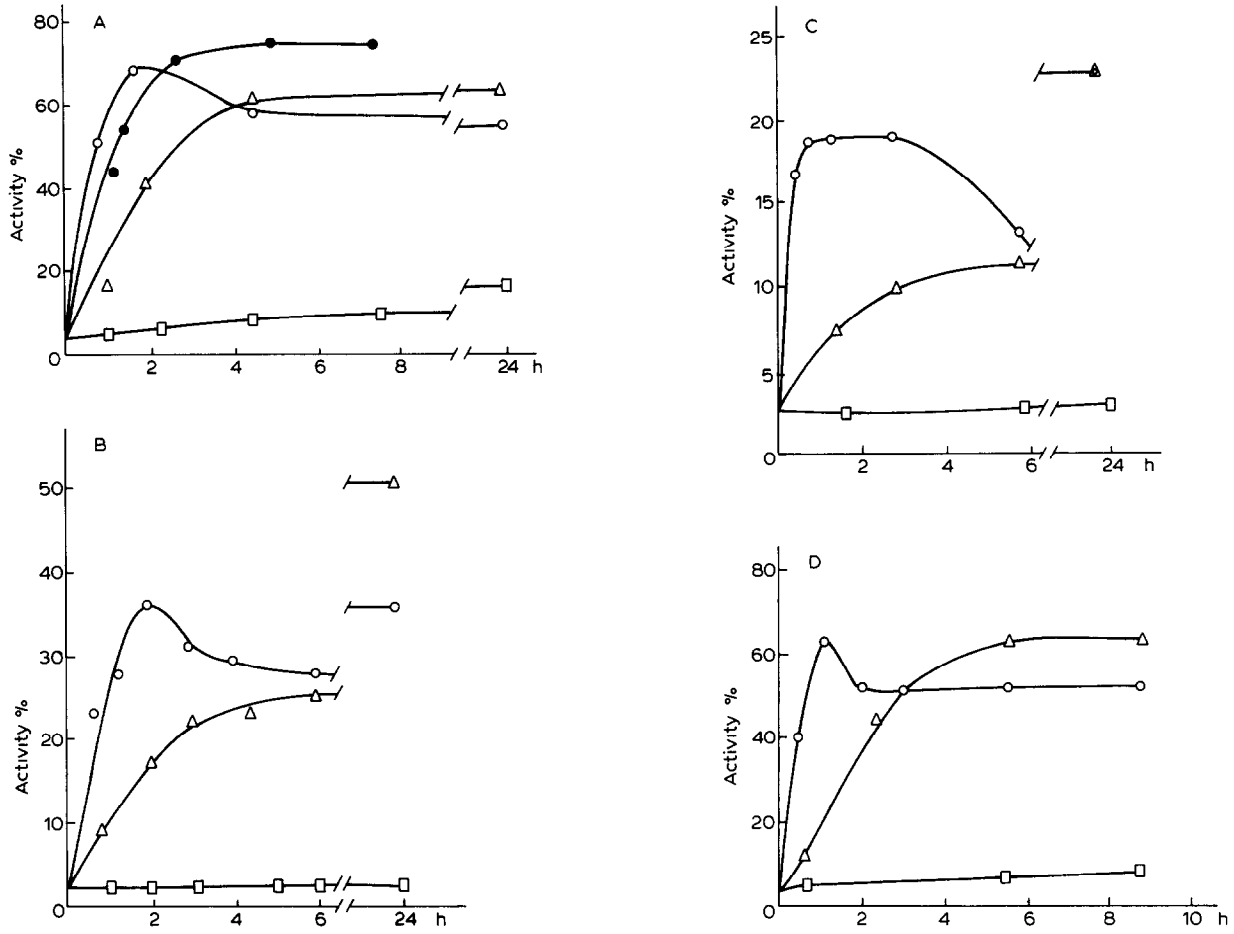

Fig.1. Prochymosin (0.2 mg/ml) activation at 37°C (□) spontaneous and by external proteinases at an enzyme:prochymosin ratio of (○) 1:100; (●) 1:250; and (△) 1:1000. The ordinates show milk clotting activity expressed as % to the maximal value attained after spontaneous activation at pH 2.0. (A) Thermolysin at pH 5.05; (B) thermolysin at pH 5.88; (C) thermolysin at pH 6.4; (D) *L. pneumophila* metalloproteinase at pH 5.05.

rather extended stretch of the zymogen polypeptide chain is available for proteolysis.

(3) It appears that the pH-dependent conformation transition starts at a higher pH for prochymosin, but at a lower pH for swine pepsinogen [5]. Anyway, the proenzyme spatial structure is well adapted to its activation by external proteinases. It is tempting to assume that this adaptation is not a matter of chance, but rather the essential trait of the proenzyme structure necessary for its intermolecular activation. In our opinion, the intermolecular activation pathway should be considered at least as equally important as the intramolecular activation pattern.

(4) Rather efficient pepsinogen and especially prochymosin activation by metalloproteinases apparently not involved in this process in vivo induces the question of the eventual role of certain 'external' proteinases that might initiate the activation process by formation of the active pepsin or chymosin.

The initiation of the activation by an external proteinase is rather common for the zymogens that belong to other classes of proteolytic enzymes. We do not imply that an externally activating proteinase should necessarily be a metalloproteinase; the most important factor governing the whole process appears to be the zymogen conformational transition exposing the crucial amino acid sequence for external proteinase action.

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