Catalytic Importance of the C-Terminal Region of a Fibrinolytic Enzyme from *Lumbricus rubellus*

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Abstract: Two fibrinolytic enzymes from the autolysate of *Lumbricus rubellus* were purified in homogeneous form. Their molecular sizes were 31,000 (Enz1) and 35,000 (Enz2) Da, respectively. However, the N-Terminal amino acid sequences of Enz1 and Enz2 were exactly the same: Ile-Val-Gly-Gly-Ile-Glu-Ala-Arg-Pro-Tyr-Glu-Phe-Pro-Trp-Gln-. These results indicate that Enz1 is a shortened form of Enz2 formed during autolysis. When a synthetic substrate, Ile-Pro-Arg-pNA, was used, the catalytic activity were observed in the pH range of 5-10 and the kinetic parameters including K_m (1.6 μM) and V_{max} (40 nmol/min/mg) were almost identical between the two enzymes. However, the fibrinolytic activity of Enz2 was at least 1.25 times higher than that of Enz1, suggesting that the C-terminal region of Enz2 is important in fibrinolysis but not in amidolysis. Furthermore, fibrinolytic activity of the enzymes was increased by the addition of the lipid extracted from *L. rubellus* in the presence of $MgCl_2$ or $CaCl_2$. The stimulatary effect of lipid on Enz2 was higher compared to Enz1.

Key words: fibrinolytic enzyme, *L. rubellus*.

The insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin which is generated from plasminogen by plasminogen activators, such as tissue plasminogen activator, vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor, and streptokinase-plasminogen complex (Collen, 1980; Collen and Lijnen, 1991). The proteolytic enzyme plasmin has a broad specificity, which is not very different from that of trypsin. However, *in vivo* the main target of plasmin is fibrin (Wiman and Collen, 1978). Fibrin markedly enhances the activation of plasminogen by activators. This seems to be due to the adsorption of both plasminogen and activators to the fibrin network. Proteinases from Lumbricus rubellus have been reported to have fibrinolytic activity, and their therapeutic use has been patented (Mihara, 1986). However, little is known about the catalytic nature of fibrin hydrolysis.

In this paper we present the evidence that the C-terminal region of a fibrinolytic enzyme from *L. rubellus* may play a role in increasing its activity.

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Materials and Methods

Materials

Fibrinogen and thrombin were purchased from Sigma Chem. Co. (St. Louis, USA). Ile-Pro-Arg-pNA was from Kabi. Benzamidine-Sepharose 6B was from Pharmacia. (Uppsala, Sweden). All chemicals were of analytical grade. *L. rubellus* were supplied by an earthworm farm, Singal, Korea.

Enzyme assay

Fibrinolytic activity: It was determined by using a fibrin plate (9 cm dia) prepared with 15 ml of 0.7% fibrinogen mixed with 7.5 units of thrombin. The enzyme solution was applied on the fibrin plate, and the plate was incubated at 37℃ for 4 h, and the clear zone was measured for relative activity. Units were calibrated with a standard plasmin.

Amidolytic activity: The enzyme in 0.1 M Tris buffer, pH 8.0, was preincubated at 37℃ for 10 min. Then chromogenic substrate, Ile-Pro-Arg-pNA, was added to make a final concentration of 10 nM in 0.5 ml of total volume. After incubating for 10 min the absorbance of the reaction mixture at 405 nm was determined (ε=10,500 $cm^{-1} \cdot M^{-1}$ for para nitroaniline (pNA)).

Preparation of crude extract

100 g of frozen *L. rubellus* was thawed in 100 ml of 10 mM sodium phosphate buffer, pH 5.0, in the presence of 0.3% benzoic acid and incubated with a gentle shaking at 30℃ for 3 days. After the incubation, the autolysate was centrifuged at 15,000×*g* for 30 min to remove debris. The supernatant was used for further purification.

Purification of fibrinolytic enzyme

The pH of the crude extract was adjusted to 4.5 by dropwise addition of 1 N acetic acid and the precipitated proteins were centrifuged out. To this enzyme solution, cold ethanol was added until the concentration of alcohol reached 60% (v/v). After removing a precipitate by centrifugation, more alcohol was added to 80% (v/v). The precipitated proteinase was collected by centrifugation, resuspended in 10 mM sodium phosphate buffer, pH 8.0, and twice dialyzed against the same buffer for 6 h. The enzyme solution obtained from the previous step was applied to the first benzamidine-Sepharose column (2.6×2.7 cm) which was pre-equilibrated with 10 mM sodium phosphate buffer, pH 8.0. The column was washed with the same buffer and subsequently with 0.1 M acetate buffer, pH 5.0. The enzyme was eluted with the same buffer containing 0.5 M arginine. The fractions having enzyme activity were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 8.0, and then applied to a benzamidine-Sepharose column (2.6×1.5 cm) pre-equilibrated with 10 mM sodium phosphate buffer, pH 8.0. The elution was performed by the same procedure described above. The concentrated enzyme in 10 mM sodium phosphate buffer, pH 8.0, was applied again on a DEAE Toyopearl 650 M FPLC column pre-equilibrated with 10 mM sodium phosphate buffer, pH 8.0. The enzymes bound on the column were eluted by applying a linear gradient of NaCl (0.04~0.36 M). The enzymes, Enz1 and Enz2 were eluted in the fractions containing 0.20 M and 0.25 M NaCl, respectively.

N-Terminal amino acid analysis

The purified enzymes were run on SDS polyacrylamide gel electrophoresis and transferred to a Immobilin-P polyvinylidene difluoride transfer membrane electrophoretically. The enzyme bands on the membrane were cut and applied to an amino acid sequencer (Milligen 6600 protein sequencer, Korea Basic Science Center).

Extraction of lipid

Lipid was extracted from *L. rubellus* with chloroform : methanol (2 : 1) (Kates, 1986). *L. rubellus* (12 g) was homogenized with chloroform:methanol (2 : 1) (90 ml), and centrifuged at 15,000×*g* for 5 min. The precipitate was suspended and homogenized with chloroform : methanol : HCl (2 : 1 : 0.8) and centrifuged at 15,000 ×*g* for 5 min. After the supernatant was neutralized with 0.2 M NH_2OH in methanol, lipid was solublized by chloroform, and it was dried by evaporator.

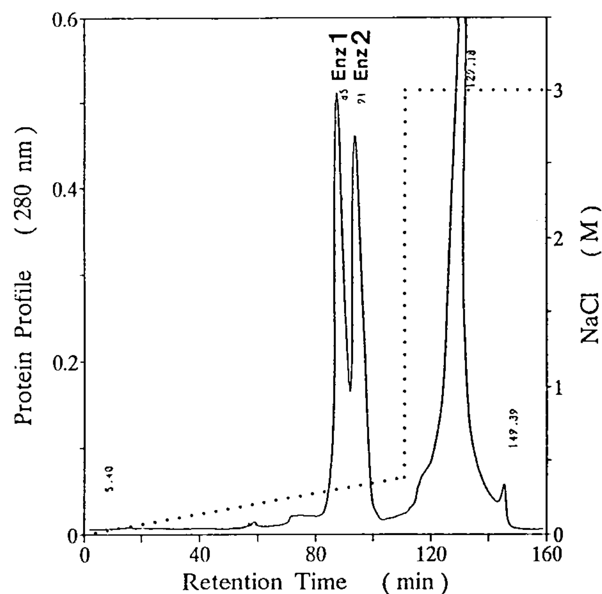

Fig. 1. DEAE ion exchange chromatography. The enzyme solution obtained from the previous step was loaded on a preparative FPLC installed with a DEAE Toyopearl 650 M column (1.2×17.5 cm) and the bound proteins were eluted by a salt gradient (0.04 ~0.36 M NaCl).

Table 1. Purification of fibrinolytic enzymes, Enz1 and Enz2, from *L. rubellus*

	Total protein (mg)	Total fibrinolytic activity (U)	Specific activity (U/mg)	Purifi. (fold)
Crude lysate	3,200	2,294	0.72	1.00
Acidification	3,150	2,082	0.66	0.92
Ethanol precipitation	262	352	1.34	1.87
1st Benzamidine sepharose	12	247	23.33	28.6
2nd Benzamidine sepharose	8	170	36.25	29.6
DEAE (FPLC) Enz1	2.1	53	25.23	35.1
Enz2	2.3	72	31.30	43.5

Results and Discussion

Purification of fibrinolytic enzymes

The fibrinolytic enzymes from *L. rubellus* were purified homogeneously by several chromatographic procedures (Table 1). *L. rubellus* were disintegrated by autol-

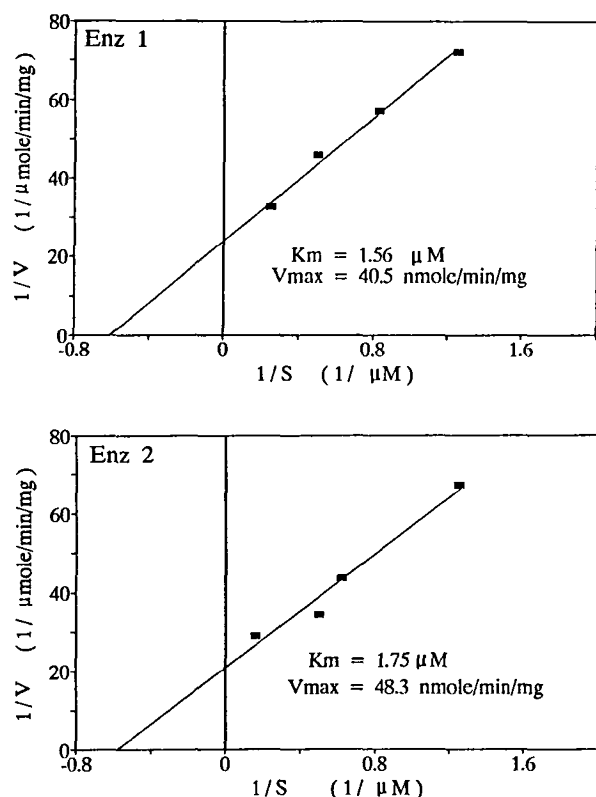

Fig. 2. The effect of concentration on the hydrolysis of Ile-Pro-Arg-pNA by the fibmolytic enzymes, Enzl and Enz2.

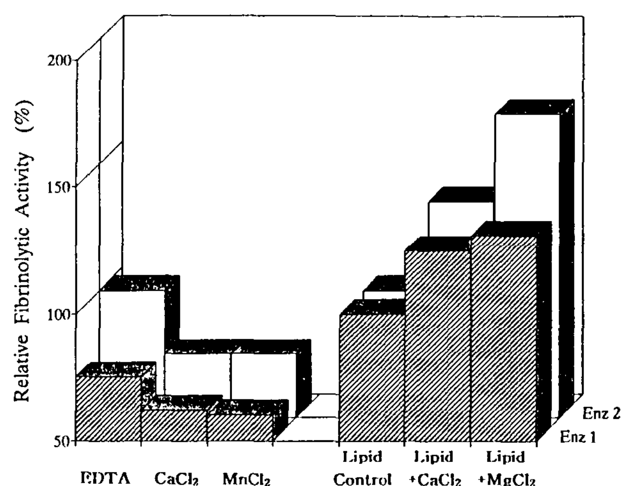

Fig. 3. The effect of salt and lipid on fibrinolysis by the enzyme, Enzl and Enz2 (▨; Enzl, □; Enz2). The final concentration of $CaCl_2$ and $MnCl_2$ were 10 mM, and lipid samples contain 0.1 mg of total lipid extracted from *L. rubellus*. The enzymes were preincubated with ions or with ions in the presence of lipid for 1 h at 37℃.

ysis when they were incubated at 30℃ for 72 h with shaking. To this lysate acetic acid was added until the pH of the solution reached 4.5. This step was not effective in increasing specific activity, but it was helpful for the final purification. After removing a precipitate from this lysate by centrifugation, fibrinolytic enzymes were collected as a second precipitate by alcohol fractionation (60~80%). This step resulted in a 1.8 fold purification. And two applications of benzamidine Sepharose chromatography resulted in about a 30-fold purification. Finally two enzymes, Enzl and Enz2, were separated by FPLC which was installed with a DEAE ion exchange column. They were eluted from the column at nearly 0.2 M NaCl when a linear gradient was applied (Fig. 1). Alcohol precipitation and repeated benzamidine sepharose chromatography were not very good methods in terms of yield or purification efficiency, but they were very useful procedures for getting a highly pure form of the two enzymes (Table 1). It was reported previously that there are at least 6 forms of fibrinolytic enzymes in *L. rubellus* (Mihara, 1986).

Properties of the fibrinolytic enzymes

The molecular sizes of the two enzymes were determined to be 32 (Enzl) and 36 (Enz2) kDa, respectively. However, the N-terminal amino acid sequences of the enzymes were identical (Ile-Val-Gly-Gly-Ile-Glu-Ala-Arg-Pro-Tyr-Glu-Phe-Pro-Trp-). This indicates that they are the same enzymes but Enzl is a shortened form of Enz2, resulting from proteolytic cleavage in the C-terminal region. Of course the possibility that they are two different enzymes having the same N-terminal sequence can not be completely ruled out. The pH optimum, 6~7.5, of the two enzymes were almost identical. The enzymes hydrolyzed Ile-Pro-Arg-pNA at the same rate and they showed the same substrate affinity (V_{max}; 40 and 48 nmol/min/mg and K_m; 1.6 and 1.7 μM, for Enzl and Enz2, respectively) (Fig. 2) These results suggest that the catalytic domain is located on the N-terminal side of the enzyme and that the C-terminal 4 kDa of Enz2 does not influence the hydrolysis of the artificial substrate. Surprisingly, however, the fibrinolytic activities of the enzymes were different (Fig. 3). The fibrinolytic activity of Enz2 was higher than that of Enzl.

The activity of the enzymes was decreased in the presence of salts, such as $CaCl_2$, and $MnCl_2$. A very similar phenomenon in interaction between thrombin and fibrin was observed (Kaminski and McDonagh, 1987). That is, the active center of thrombin associated with fibrin through extended binding is fully exposed and freely accessible. For fibrin, a single class of binding sites was observed and the binding was inversely related to the ionic strength. Increasing ionic strength releases thrombin from its binding domain on the fibrin monomer (Liu *et al.*, 1979; Kaminski and McDonagh, 1983). Considering that the results reported previously the fibrinolytic enzyme from *L. rubellus* may have a

binding site, independent of the catalytic center. The binding site may be localized on the C-terminal side of the enzyme. The increase in the fibrinolytic activity of the enzymes was observed by the addition of neutral lipids extracted from *L. rubellus*, as well as detergent in the presence of $MgCl_2$ or $CaCl_2$. In this case the increasing effect of Enz2 was also higher than that of Enz1. The experiments with polar phospholipids did not show consistent results (data not shown). These results suggest that the lipids in the presence of salts may influence the affinity of the fibrinolytic enzyme to fibrin. The interaction of a fibrinolytic enzyme with an insoluble fibrin clot seems to be a dynamic process which involves recognized-adsorption, catalysis, and desorption on the fibrin surface. In addition to the catalytic events, the binding of the enzyme onto the heterogeneous surface of a fibrin clot is also important in increasing its hydrolytic activity. At present nothing is clearly known about the multidomain structure of the fibrinolytic enzyme from *L. rubellus*. But the evidence presented here suggests the possibility that an effector, maybe a binding site or domain, is located in the C-terminal region of the fibrinolytic enzyme, whereas the catalytic domain is located on the N-terminal side.

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