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A NEW POLYPEPTIDE SUBSTRATE, SUC-TYR-LEU-VAL-pNA, SPECIFIC FOR SPLEEN FIBRINOLYTIC PROTEINASE (SFP)

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Summary: In a search for an adequate synthetic substrate for human spleen fibrinolytic proteinase (SFP), Suc-Tyr-Leu-Val-pNA was newly synthesized. Its degradation by SFP was compared with that of other synthetic polypeptide substrates. Suc-Tyr-Leu-Val-pNA was degraded by SFP with a high K_{cat}/K_m, but it was not degraded by the following serine proteinases, plasmin, trypsin, thrombin, chymotrypsin, urokinase and milk plasminogen-activator, or by pancreatic elastase to any practical extent.

INTRODUCTION

An insoluble neutral proteinase capable of degrading fibrin and fibrinogen was purified by us from the human spleen in a previous study (1). Most of its examined physicochemical and enzymatic properties resembled those of elastases except for the substrate specificity (1, 2). An adequate synthetic substrate for the enzyme has not yet been obtained, although a synthetic substrate was considered necessary for promoting further studies. In his work on leucocyte elastase, Blow (3) reported that elastase cleaves between Val-12 and Glu-13, and between Val-18 and Cys (O_3H)-19 of the B-chain of insulin. Suc-Tyr-Leu-Val-pNA and Ac-Ser-His-Leu-Val-pNA were therefore synthesized, imitating the amino acid sequence in the B-chain of insulin, and the amidolysis by SFP was studied.

MATERIALS AND METHODS

SFP was purified from human spleen by ultracentrifugation, chloroform treatment, salting out with 50% saturated ammonium sulfate and repeated Sephadex G-100 gel chromatography. The resultant preparation showed a single protein band possessing fibrinolytic activity on polyacrylamide gel electrophoresis at pH 4.5 (1). Pancreatic elastase (porcine, type III) was purchased from Sigma Chem. Co. (St. Louis) and dissolved in Tris-HCl buffer (0.1 M, pH 8.0) containing 2 M $NaClO_4$. The same buffer was used to dilute all enzymes. Trypsin (trypure, Novo, Copenhagen),

Abbreviations: Suc, succinyl; pNA, p-nitroanilide; Ac, acetyl; t-Boc, tert-butyloxycarbonyl; Z, benzyloxycarbonyl; DCC, N, N'-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DPPA, diphenylphosphorazidate; PCA, perchloric acid; Plg, plasminogen

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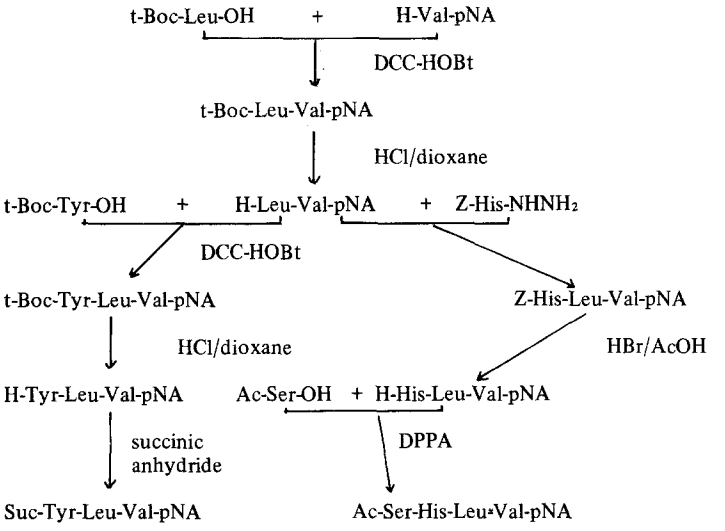

Fig. 1 Synthetic routes to the two substrates

chymotrypsin (Miles Lab., Erkhart), thrombin (Mochida, Tokyo), urokinase (Uronase, Mochida, Tokyo) and milk Plg-activator purified in our laboratory (4) were employed. Human plasminogen (KABI, Stockholm) activated by urokinase was used for plasmin.

Fibrinogen (KABI, Stockholm) and elastin (Nakarai, Kyoto) were employed as protein substrates apart from fibrin. Suc-Ala_3-pNA and Suc-Ala-Pro-Ala-pNA were purchased from the Protein Research Foundation (Osaka).

Suc-Tyr-Leu-Val-pNA and Ac-Ser-His-Leu-Val-pNA were synthesized in our laboratory by the routes shown in Fig. 1. For the synthesis of the key intermediate, H-Leu-Val-pNA, t-Boc-Leu-OH and H-Val-pNA were coupled using the DCC-HOBt method (5) followed by treatment with HCl in dioxane. t-Boc-Tyr-OH was coupled with the H-Leu-Val-pNA by the DCC-HOBt method to give t-BOC-Tyr-Leu-Val-pNA. After removal of N-protecting group by HCl in dioxane, the resulting amine was acylated with succinic anhydride (6) in AcOEt to give Suc-Tyr-Leu-Val-pNA. The peptide was purified by silica gel column chromatography using 1% MeOH in $CHCl_3$ as the eluent. The material was homogeneous upon thin-layer chromatography on silica gel G ($CHCl_3$-MeOH-H_2O=90:8:3, Rf 0.83; $CHCl_3$-MeOH-H_2O=8:3:1, Rf 0.48) The other substrate, Ac-Ser-His-Leu-Val-pNA, was prepared as follows. Z-His-$NHNH_2$ was coupled with H-Leu-Val-pNA by the azide procedure (7) to give Z-His-Leu-Val-pNA. The Z group was removed with HBr-AcOH and the resultant amine was coupled with Ac-Ser-OH by the DDPA method (8) yielding Ac-Ser-His-Leu-Val-pNA. This was purified by column chromatography on silica gel, eluting with $CHCl_3$, MeOH and H_2O (16:3:1), TLC, Rf 0.50 ($CHCl_3$-MeOH-H_2O=8:3:1). Full details of the synthesis and characterization of these compounds are to be published elsewhere (Y. Okada et al., in preparation). Ten μM of Suc-Tyr-Leu-Val-pNA and Ac-Ser-His-Leu-Val-pNA dissolved in diethylene dioxide (1, 4-dioxane, $C_4H_8O_2$) were added to 19 volumes of Tris-HCl buffer (0.1 M, pH 8.0), and further diluted with the buffer to give the required concentration.

Fibrinolytic activity was measured on Plg-free fibrin plates (2). Elastolytic activity was measured on elastin-agar plates and SDS elastin-agar plates (9, 10). Fibrinogenolytic activity was assayed by measuring the PCA soluble products (E_{280}). Amidolytic activity was assayed by measuring the released pNA (E_{410}). Michaelis constants were calculated from Lineweaver-Burk plots at a substrate concentration of 0.05–3.0 mM.

RESULTS AND DISCUSSION

The SFP preparation was diluted to give the same fibrinogenolytic activity as the pancreatic elastase used. The relative sensitivities of both enzymes for the protein and synthetic substrates were then compared.

The data for protein substrates are summarized in table 1. As shown in the table, pancreatic elastase degraded elastin and fibrin well. However, SFP degraded fibrin well, but not elastin even in the presence of SDS. Higher concentrations of SFP never degraded elastin in other experiments. These results indicate that SFP has a different substrate specificity from elastase.

Table 2 shows the comparative amidolytic activity of SFP and elastase against certain polypeptide substrates. Among the substrates examined, SFP most easily amidolysed Suc-Tyr-Leu-Val-pNA, whereas elastase degraded Suc-Ala_3-pNA and Suc-Ala-Pro-Ala-pNA well.

The kinetic parameters of SFP and elastase for the substrates used in the experiments in table 2 were then estimated. The data obtained are listed in table 3.

The highest value of K_{cat}/K_m for SFP was obtained with Suc-Tyr-Leu-Val-pNA, whereas that for elastase was obtained with Suc-Ala-Pro-Ala-pNA. Suc-Tyr-Leu-Val-pNA was a far more specific substrate for SFP than for elastase.

The degradation of Suc-Tyr-Leu-Val-pNA by various serine proteinases was examined. The results (table 4) indicated that the polypeptide substrate was not degraded to any practical extent by pancreatic elastase, and was not degraded at all by the other proteinases examined apart from SFP.

The experiments summarized in tables 2–4 indicate that Suc-Tyr-Leu-Val-pNA may be a highly specific synthetic substrate for SFP, and its application may be helpful for the characterization of SFP.

In leucocyte granules three neutral proteinases, collagenase (11–14), elastase (12, 14–17) and cathepsin G (chymotrypsin-like enzyme) (16, 18–20), have been reported by various workers. In our studies, Suc-Tyr-Leu-Val-pNA was found to be slightly degraded by human leucocyte extract in neutral medium without the addition of metal ions. This suggests that the Suc-Tyr-Leu-Val-pNA degradable enzyme may not be leucocyte collagenase. Since chymotrypsin did not degrade the synthetic substrate at all, the leucocyte enzyme is not considered to be cathepsin G. Further studies on the degradation of Suc-Tyr-Leu-Val-pNA by purified leucocyte enzymes are required.

Table 1 Comparative proteolytic activity of SFP and elastase

	SFP	Elastase
Fibrinogen degradation (E_{280})*	0.2	0.2
Plg-free fibrin plates (mm^2)**	530.0	210.0
Elastin-agar plates (mm^2)**	0	36.0
SDS elastin-agar plates (mm^2)**	0	180.0

* The final concentration of fibrinogen was 2.5%, and those of SFP and elastase were 4 μg/ml and 2 μg/ml, respectively, in the reaction mixture. Incubation was performed at 37°C for 15 min.

** Lysis areas on fibrin and agar plates after incubation for 20 hr at 37°C. The volume of enzyme solution placed on the plates was 0.03 ml.

Table 2 Comparative amidolysis of SFP and elastase

Substrate	Released pNA (μM)/mg enzyme/10 min	
	SFP	Elastase
Suc-Ala_3-pNA	4.29	72.5
Suc-Ala-Pro-Ala-pNA	13.1	160.0
Suc-Tyr-Leu-Val-pNA	45.0	0.37
Ac-Ser-His-Leu-Val-pNA	20.6	0.1

5×10^{-4} M substrate and various amounts (2–200 μg) of enzyme were incubated for 10 min. The released pNA was estimated and the activity was expressed as pNA (μM)/mg enzyme/10 min.

Table 3 Kinetic parameters for the amidolysis of certain synthetic substrates by SFP and elastase

Substrate	Enzyme	K_m(mM)	$K_{cat}(s^{-1})$	$K_{cat}/K_m(M^{-1}s^{-1})$
Suc-Ala_3-pNA	SFP	1.7	0.83	488
	Elastase	2.0	8.0	4,000
Suc-Ala-Pro-Ala-pNA	SFP	2.5	3.14	1,256
	Elastase	3.0	31.0	10,333
Suc-Tyr-Leu-Val-pNA	SFP	0.17	3.85	22,647
	Elastase	10.0	0.21	21
Ac-Ser-His-Leu-Val-pNA	SFP	1.0	2.7	2,700
	Elastase	17.5	0.15	9

The K_{cat} of SFP was calculated, assuming that all protein in the purified preparation was SFP.

Table 4 Comparison of the amidolysis of Suc-Tyr-Leu-Val-pNA by various proteinases

Enzyme	Released pNA (μM)/mg enzyme/10 min
SFP	45.0
Elastase	0.37
Plasmin	0
Trypsin	0
Chymotrypsin	0
Thrombin	0
Urokinase	0
Plg-activator (Milk)	0

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