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Chromogenic Substrates for Horseshoe Crab Clotting Enzyme

Its Application for the Assay of Bacterial Endotoxins[1]

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Abstract. An endotoxin-activated hemocyte lysate from the horseshoe crab (*Tachypleus* and *Limulus*) was found to hydrolyze Bz-Ile-Glu-(γ-OR)-Gly-Arg-*p*-nitroanilide (PNA), Bz-Val-Gly-Arg-PNA, Boc-Val-Leu-Gly-Arg-PNA, and Boc-Leu-Gly-Arg-PNA, all of which have the COOH-terminal Gly-Arg sequence. This amidase activity was due to a clotting enzyme contained in the lysate. Furthermore, the amidase activity increased by increasing the concentration of bacterial endotoxin (*E. coli,* 0111-B4) added to the lysate. Therefore, the measurement of the endotoxin-induced amidase activity made it possible to determine the concentration of the endotoxin.

Introduction

A hemocyte lysate from horseshoe crab contains a pro-clotting enzyme, which is transformed to the active clotting enzyme in the presence of gram-negative bacterial endotoxin (1–3). The clotting enzyme coagulates a clottable protein, named coagulogen, which is also contained in the hemocyte lysate (4, 5). This gelation reaction of the lysate, so-called *Limulus* test, has been widely employed as a simple and very sensitive assay method for endotoxin (6, 7). In a

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previous paper (8), we found that the gelation of coagulogen by the endotoxin-induced clotting enzyme involves a limited proteolysis. This indicates that the clotting enzyme must have a proteolytic or esterolytic activity like α-thrombin. We wish to report here further investigations on substrate specificity of the clotting enzyme and a new sensitive assay method for bacterial endotoxin.

Materials and Methods

Horseshoe Crab Hemocyte Lysate

The hemocyte lysate from Japanese horseshoe crab *(Tachypleus tridentatus)* was prepared by the method previously described (9). *Limulus polyphemus* lysate was a generous gift from Dr. *E.B. Seligmann,* jr., Bureau of Biologics, FDA, Bethesda, Md. A commercial preparation of hemocyte lysate from *Tachypleus tridentatus,* named Pregel® (Seikagaku Kogyo Co. Ltd., Tokyo), was also used.

*Peptide-*p*-Nitroanilide (PNA) Substrates*

The synthetic substrates used (table I) were products of Kabi AB, Mölndal, Sweden, except Z-Gly-Pro-Arg-PNA and Bz-Val-Gly-Arg-PNA, which were obtained from Boehringer, Mannheim and Pentapharm AG, Basel, respectively, and Boc-Val-Leu-Gly-Arg-PNA, Boc-Leu-Gly-Arg-PNA, Boc-Val-Ser-Gly-Arg-PNA, and Boc-Ser(O-Bz)-Gly-Arg-PNA which were newly synthesized in our laboratory. The purity of all intermediates and final products was confirmed by thin-layer chromatography, elemental analysis, and amino acid analysis. Bz-Tyr-PNA, BAEE, TAME, acetyl-Gly-Lys-methylester, and Bz-Arg-β-naphthylamide were products of the Protein Research Foundation, Minoh, Osaka. Most of the synthetic substrates were dissolved in a pyrogen-free distilled water, but some of the water-insoluble peptides were dissolved in dimethylsulfoxide or dimethylformamide and diluted with Tris-HCl buffer used for the enzyme assay.

Preparation of Endotoxin Solution

A lipopolysaccharide prepared from *E. coli* 0111-B4 was a product of Difco Laboratories and used as a standard endotoxin. Heptose-free glycolipid was prepared from *Salmonella minnesota* R595 (10). The endotoxin was dissolved in a pyrogen-free saline.

Assay Procedure of Clotting Enzyme

As the source of clotting enzyme, the hemocyte lysates prepared from *Tachypleus tridentatus* and *Limulus polyphemus,* and Pregel were used. The reaction mixture containing 0.8 ml of 0.1 m*M* synthetic substrate dissolved in 0.1 *M* Tris-HCl buffer, pH 8.0, 50 μl of 0.5 *M* $MgCl_2$, and 20 μl of a 0.1% endotoxin solution (*S. minnesota* R595) was preincubated at 37 °C for 3 min. Then, 20–50 μl of the lysate was added and the mixture incubated further for 15 min. After incubation, 100 μl of glacial acetic acid was added to terminate the reaction and the PNA released was measured spectrophotometrically at 405 nm.

Table I. Hydrolysis of various synthetic substrates by horseshoe crab hemocyte lysates

Substrates	PNA released, nmol/15 min	
	Tachypleus	*Limulus*
Bz-Ile-Glu(γ-OR)-Gly-Arg-PNA	17.7 (100)	4.4 (100)
Bz-Val-Gly-Arg-PNA	12.4 (70)	10.9 (227)
Boc-Val-Leu-Gly-Arg-PNA	11.0 (62)	19.1 (431)
Boc-Leu-Gly-Arg-PNA	17.7 (100)	7.7 (175)
Boc-Val-Ser-Gly-Arg-PNA	8.3 (47)	10.9 (227)
Boc-Ser(OBz)-Gly-Arg-PNA	19.6 (111)	42.7 (971)
Bz-Phe-Val-Arg-PNA	0.6 (3)	0.2 (5)
Z-Gly-Pro-Arg-PNA	0.4 (3)	0.4 (9)
H·D-Phe-Pip-Arg-PNA	–	0.3 (6)
H-Glu-Gly-Arg-PNA	<0.2	<0.1
H·D-Val-Leu-Arg-PNA	<0.2	<0.1
H·D-Val-Leu-Lys-PNA	<0.2	<0.1
H·D-Pro-Phe-Arg-PNA	<0.2	<0.1
Bz-Tyr-PNA	ND	–
Acetyl-Gly-Lys-Me	4.6 μmol/min/A_{280} = 1.0	
TAME	ND	

Numbers in parentheses are the percent relative activity.
Bz = Benzyl; Boc = tert-butoxycarbonyl; Z = carbobenzoxy; H·D = *D*-amino acid residue; Pip = pipecolyl; Acetyl-Gly-Lys-ME = α-N-acetyl-glycyl-lysine methyl ester; TAME = α-N-tosyl-arginine-methyl ester; ND = not detectable.

Results and Discussion

Table I shows the amidase acivities of *Tachypleus* and *Limulus* lysates towards various anilide substrates, which were recently introduced as chromogenic substrates for blood clotting factors and their related enzymes (11). Both lysates showed activity towards the anilide substrates for factor Xa (Bz-Ile-Glu[γ-OR]-Gly-Arg-PNA) and for urokinase (Bz-Val-Gly-Arg-PNA). Moreover, new anilide substrates, Boc-Val-Leu-Gly-Arg-PNA, Boc-Leu-Gly-Arg-PNA and Boc-Ser(0-Bz)-Gly-Arg-PNA were easily hydrolyzed. The tri- and tetrapeptide sequences of these substrates originate from the sequences located close to the sites cleaved during the gelation of coagulogen by *Tachypleus* clotting enzyme (12, 13). On the other hand, the substrates, which have been used for α-throm-

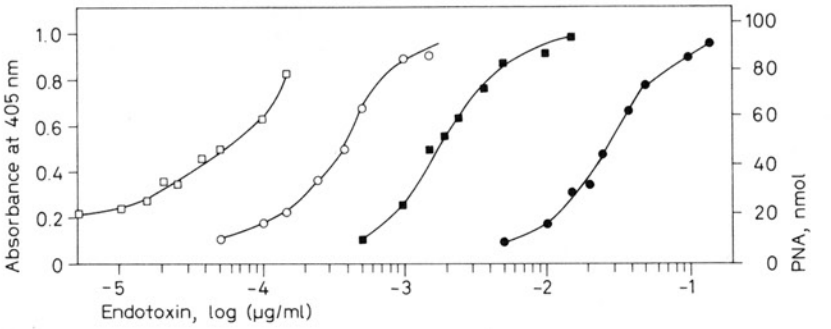

Fig. 1. Effect of different concentrations of *E. coli* 0111-B4 endotoxin on amidase activity of Pregel (*Tachypleus* hemocyte lysate). Sterilized and pyrogen-free glass ware and reagents were used. The final concentration of the substrate, Boc-Val-Leu-Gly-Arg-PNA, was 0.5 m*M*. The reaction mixtures (described in text) were incubated at 37 °C for 15 min (•), 30 min (▪), 60 min (○), and 240 min (□).

bin (Bz-Phe-Val-Arg-PNA, Z-Gly-Pro-Arg-PNA, and H·D-Phe-Pip-Arg-PNA), kallikreins (H·D-Val-Leu-Arg-PNA and H·D-Pro-Phe-Arg-PNA), plasmin (H·D-Val-Leu-Lys-PNA), and urokinase (H·Glu-Gly-Arg-PNA), were unsusceptible to both lysates. In addition, *Tachypleus* lysate showed little activity towards Bz-Tyr-PNA, TAME, and Bz-Arg-β-naphthylamide under the same conditions as used for the oligopeptide substrates (table I).

These results clearly indicate that horseshoe crab clotting enzyme displays a high specificity towards the peptide PNA having the COOH-terminal Gly-Arg sequence. The K_m values estimated by the Lineweaver-Burk plots for Tos-Ile-Glu-Gly-Arg-PNA, Boc-Val-Leu-Gly-Arg-PNA, and Bz-Val-Gly-Arg-PNA, using a partially purified *Tachypleus* clotting enzyme, were 2.1×10^{-4} *M*, 5.4×10^{-5} *M*, and 4.6×10^{-5} *M*, respectively.

Figure 1 shows the effect of the concentration of bacterial endotoxin on the amidase activity of commercial *Tachypleus* lysate, Pregel. In this experiment, the reaction mixture containing 50 μl of 2 m*M* Boc-Val-Leu-Gly-Arg-PNA dissolved in pyrogen-free distilled water, 100 μl of endotoxin solution (*E. coli* 0111-B4), 50 μl of Pregel solution (one ampule Pregel was dissolved in 250 μl of 0.4 *M* Tris-HCl buffer, pH 8.0, containing 0.04 *M* $MgCl_2$), in a total volume of 200 μl, was incubated at 37 °C. After incubation for indicated times, 0.8 ml of 12.5% glacial acetic acid was added to terminate the reaction and the PNA released was measured. The result shown in figure 1 indicates that the amidase activity of the lysate increases with increasing concentration of endotoxin and that a sigmoid

relationship between the toxin concentration in logarithm and the activity can be observed in the range of 5×10^{-6} to 5×10^{-2} μg endotoxin. Thus, the measurement of amidase activity induced in the hemocyte lysate by endotoxin can be applicable for the detection and the quantitation of bacterial endotoxins. The method is a ten-times more sensitive than that of the *Limulus* gelation test and is very reproducible.

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