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Dog Pancreatic Arginine Esterases, spontaneously activated on DEAE-Sephadex

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Tremendous activation of arginine esterases or trypsin-like enzymes from the dog pancreas was observed during DEAE-Sephadex A-50 chromatography of the pancreatic kallikrein. The esterase activity freshly assayed was 7.5 μmoles N^{α}-benzoyl-L-arginine ethyl ester (BAEE)/min per gram of the pancreas. The enzymes were separated into two fractions by Ampholine isoelectric focusing, their isoelectric points being 4.6 and 4.8. The two esterases purified showed the specific activities with 4.4—7.4 μmoles BAEE/min/A_{280} and 20—36 μmoles N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester (TAME)/min/A_{280}, and hydrolyzed N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) and casein. The esterases were strongly inhibited by Trasylol, soybean trypsin inhibitor, kallikrein inhibitors from potatoes, *etc.* From chemical and enzymatic properties, both esterases seemed to be anionic trypsins of the dog pancreas.

In the research of plasma kallikrein[2)] and glandular kallikrein,[3)] the presence or activation of arginine esterases or trypsin-like enzymes sometimes has induced troublesome problems. Enzymatic properties of both kallikrein and trypsin are similar to each other in some respects. Glandular kallikreins are generally acidic proteins with molecular weights around 30000.[4)] The separation of the above esterases or trypsin-like enzymes from kallikrein would be an important point for the identification of kallikrein. Further, they should be checked up in the enzymatic analysis of some diseases like pancreatitis[5)] to which kallikrein–kinin system has been related.

During the studies on dog pancreatic kallikrein (Dog Panc. K.),[6)] we encountered a tremendous, unexpected activation of arginine esterases on DEAE-Sephadex A-50. Two of these dog pancreatic esterases (DPE) were found to exist and purified by isoelectric focusing, showing remarkably different properties from those of Dog Panc. K. Both DPE hardly showed vasodilator activity, and were strikingly inhibited by some trypsin inhibitors. Thus, the properties of DPE were in contrast with those of Dog Panc. K. The possibility of their identification with the anionic trypsin was also discussed in this paper.

Material and Method

Assay Methods of Enzyme——Dog vasodilator activity was measured comparing the response of sample

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2) M.E. Webster, *Fed. Proc.*, **27**, 84 (1968); M. Yano, S. Nagasawa and T. Suzuki, *J. Biochem.* (Tokyo), **67**, 713 (1970); H. Moriya and Y. Hojima, "Advances in Experimental Medicine and Biology," Vol. 21, ed. by N. Back and F. Sicuteri, Plenum Press, New York, N.Y., 1972, p. 167.
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to that of standard kallikrein (hog pancreatic) as described previously.[7a] Esterolytic activities against N^{α}-benzoyl-L-arginine ethyl ester (BAEE, at 253 nm, purchased from Protein Research Foundation, Minoh, Osaka),[7b] N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester (TAME, at 247 nm, Calbiochem.),[7c] and N-benzoyl-L-tyrosine ethyl ester (BTEE, at 256 nm, Calbiochem.)[7c] were determined at 25° and pH 8.0 according to the respective spectrophotometric methods. Hydrolysis of N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA, at 405 nm, Sigma)[7d] was also determined at 25°, pH 8.0. These assays were usually done in the absence of calcium ions. Caseinolytic activity was determined at 35° and pH 8.0,[7e] using Hammarsten casein (E. Merck AG) dissolved in 0.1 M Tris–HCl, pH 8.0.

Isoelectric Focusing——Ampholine isoelectric focusing was carried out following the method of Vesterberg and Svensson,[8] using a column of 440 ml capacity from LKB-Produkter AB.

Inhibitors——A commercial preparation of bovine kallikrein-trypsin inhibitor,[9a] Trasylol, was kindly supplied from Bayer Pharmaceutical Co., and soybean-, lima bean-, and egg white-trypsin inhibitors (SBTI, LBTI and EWTI, respectively) were purchased from Sigma. Two kallikrein inhibitors from potatoes (PKI-56 and -64) were prepared in our laboratory.[8b,9b]

Result and Discussion

Preparation of DPE

The pancreas from mongrel dogs, 171 g, was minced and homogenized with 850 ml of distilled water. The homogenate was stirred for 7 hr at room temperature, and was centrifuged to remove insoluble materials. The supernatant of 855 ml (88.9 A_{280}, 11.3 kallikrein units/ml, 0.31 μmoles BAEE/min/ml, kallikrein unit is abbreviated as KU in the following) was submitted to acetone fractionation in an ice bath. The precipitate formed at 50% (v/v)

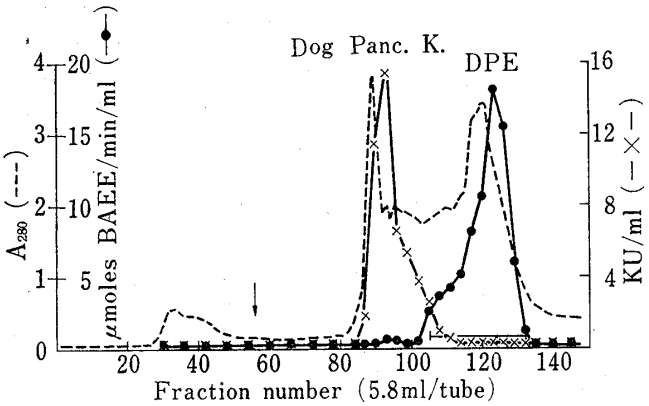

Fig. 1. Spontaneous Activation of DPE during a DEAE-Sephadex A-50 Chromatography

sample, precipitate formed between 50 and 67% (v/v) concentration of acetone of the extract from dog pancreas; column, 2.5 × 34 cm of DEAE-Sephadex A-50 equilibrated with 0.1 M Tris-HCl–0.1 M NaCl (pH 8.0); eluant, changed to 0.1 M Tris-HCl–0.3 M NaCl (pH 8.0) at ↓; temperature, 4°. Bracketed fractions were pooled and used in the next step.

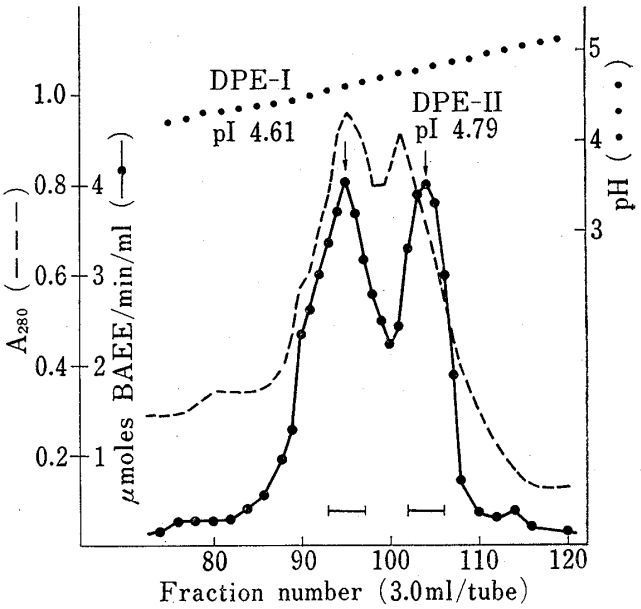

Fig. 2. Ampholine Isoelectric Focusing of DPE

sample, concentrate of the pooled solution of Fig. 1; Ampholine (LKB-Produkter AB), pH 3—5, final 1% (w/v); electrophoresis, 500 V, 41 hr, 4°. Bracketed fractions were pooled separately and filtered through a Sephadex G-25 column.

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concentration of acetone was centrifuged off, and that formed between 50 and 67% (v/v) was collected. Then the precipitate was dissolved in 15 ml of 0.1M Tris–HCl (pH 8.0) containing 0.1M NaCl, and dialyzed overnight against the buffer at 4°. In the next, the dialysate (19.3 ml, 45.0 A_{280}/ml, 79.4 KU/ml, 2.36 μmoles BAEE/min/ml) was applied to a column of DEAE-Sephadex A-50 (Pharmacia) equilibrated with the same buffer. This chromatography was developed at 4° by stepwise elution of making the NaCl concentration of the buffer increase (Fig. 1). The recovery of vasodilator activity after the chromatography was 65% against the activity of the dialysate, but that of the BAEE hydrolytic activity was really 2940%. As shown in Fig. 1, the fractions containing Dog Panc. K. were weak in BAEE hydrolysis, whereas the effluent around fraction 120 was very weak in vasodilator activity and strong in BAEE hydrolysis. It was therefore considered that large amounts of arginine esterases were newly activated in this step. The activity of 7.5 μmoles BAEE/min was freshly obtained from the pancreas equivalent to 1 g.

Fractions 105—132 (Fig. 1) were combined (135 ml, equivalent to 142 g of the starting pancreas) and concentrated to 12.4 ml (12.9 A_{280}/ml, 2.3 KU/ml, 48.3 μmoles BAEE/min/ml) at less than 10° by an Amicon ultrafiltration cell (model 402) with a UM-10 membrane (Amicon Corp.). The sample solution was then applied to Ampholine isoelectric focusing (Fig. 2). Two peaks of esterolytic activity were found at the positions of isoelectric points (pI) 4.61 and 4.79. Five fractions between Nos. 93—97 and between Nos. 102—106 were pooled separately (named DPE-I and -II respectively), and filtered through a column (2.5×43 cm) of Sephadex G-25 (Pharmacia) equilibrated with 0.005M ammonium formate (pH 5.8) at 4°. After BAEE assay, active fractions with high specific activity were pooled, and stocked at −20°.

Some Properties of DPE-I and -II

Substrate specificities of these esterases are summarized in Table I, comparing with those of Dog Panc. K.[6] The esterases showed considerably strong activities towards arginine esters and casein in contrast with their negligible vasodilator activities. Their activities to TAME were stronger than those to BAEE. These specificities of DPE were quite different from that of Dog Panc. K. (Table I).

TABLE I. Substrate Specificities of DPE-I and -II

Enzymes	KU/A_{280}	μmoles/min/A_{280}[a]					Kunitz's[b] unit/A_{280} Casein
		BAEE	BAEE (Ca^{2+})[c]	TAME	BA*p*NA	BTEE	
DPE-I	0.6	4.4	4.4	20	0.45	ND[d]	0.29
DPE-II	0.8	7.4	7.7	36	0.91	ND	0.57
Dog Panc. K.[e]	867	18.3	—	10	ND	0.05	ND

a) determined at 25°, pH 8.0
b) determined at 35°, pH 8.0
c) The concentration of calcium ions was 0.019M in the reaction mixture.
d) not detectable
e) The activities of Dog Panc. K. have been expressed with values per mg protein. Cited from our report.[6]

As shown in Table II, DPE-I and -II were strongly inhibited by four trypsin inhibitors and even by two PKI's, and the inhibition profile appeared to be proportional up to about 80% inhibition in some cases. Dog Panc. K. was not inhibited by those trypsin inhibitors.[6] However, inhibitory effects on DPE (0.84 μmoles BAEE/min/ml preincubation mixture) of synthetic inhibitors, diisopropylfluorophosphate (DFP, 10^{-3}M), N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK, 10^{-3}M) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK, 10^{-3}M),

Table II. Inhibitory Effects of Proteinase Inhibitors on DPE-I and -II[a]

Inhibitors (KIU[b] or μg/ml of preincub. mix.)		Inhibition (%) DPE-I	DPE-II	Inhibitors (μg/ml of preincub. mix.)		Inhibition (%) DPE-I	DPE-II
Trasylol	2.5 KIU	6	16	EWTI	5 μg	21	20
	10	26	64		20	78	74
	50	96	94		100	96	96
	250	100	98		500	100	98
SBTI	5 μg	44	44	PKI-56	5 μg	33	42
	20	91	96		20	89	88
	100	98	98		100	96	96
	500	100	98		500	96	96
LBTI	5 μg	89	78	PKI-64	5 μg	48	48
	20	98	94		20	87	90
	100	100	96		100	93	96
	500	100	98		500	96	98

a) Equal volumes of DPE and each inhibitor (4 graded concentrations, dissolved in 0.1M Tris–HCl, pH 8.0) or the same buffer for control were mixed and preincubated for 20 min at 37°. Then the remaining activity was determined by BAEE assay. The control tubes of DPE-I and -II showed the activities of 0.35 and 0.39 μmoles BAEE/min/ml respectively.
b) kallikrein inhibitor unit

were very weak or negligible. DPE had the function of auto-digestion at neutral pH range, although Dog Panc. K. did not.[6] The molecular weights of both DPE have been estimated to be 16000—20000 by the gel filtration technique with Sephadex G-75 and G-100 (mol. wt. of Dog Panc. K.: 28000).[6] In respect of the activation mechanism of DPE, we speculate that a mask substance covering their active sites was removed from them by the DEAE-Sephadex A-50 chromatography, because they were strongly inhibited by the filtrate which was not adsorbed on a DEAE-Sephadex A-50 column equilibrated with the first buffer 0.1M Tris–HCl–0.1M NaCl, pH 8.0. (On the other hand, during the purification of Dog Panc. K. by preparative disc electrophoresis,[6] we did not observe the spontaneous activation of DPE.)

Although calcium ions were almost uneffective on the BAEE hydrolytic activity of DPE (Table I), and DFP and TLCK were hardly inhibitory, DPE seemed to be anionic trypsins from the above results and by the additional, following reasons. The pI's of DPE-I and -II were 4.6 and 4.8 respectively (Fig. 2), and these values are in good agreement with those of a dog anionic trypsin (pI 4—5)[10] and a human anionic trypsin (pI 4.6—6.5).[11] Moreover, the former trypsin was strikingly inhibited by EWTI, and the latter by SBTI and LBTI.

In summary, tremendous activation of arginine esterases of dog pancreas was found in a DEAE-Sephadex A-50 chromatography, and two esterases with pI's 4.6 and 4.8 were guessed to be anionic trypsins from their chemical and enzymatic properties.

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