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Isolation of Dog, Rat and Hog Pancreatic Kallikreins by Preparative Disc Electrophoresis and Their Properties

Yoshio Hojima, Chiaki Moriwaki and Hiroshi Moriya

Faculty of Pharmaceutical Sciences Science University of Tokyo[1]

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Dog, rat and hog pancreatic kallikreins were efficiently purified to homogeneous forms by combining preparative disc electrophoresis with acetone fractionation and Sephadex gel filtration. The recoveries of these kallikreins through the whole purification procedures were in the range of 20—45% in vasodilator and N^{α}-benzoyl-L-arginine ethyl ester (BAEE) assays. The purified kallikreins appeared to be homogeneous in analytical disc electrophoresis; however, isoelectric focusing made it clear that they consisted of several kallikrein multiple forms with slightly different isoelectric points (pI's). The pI's of these kallikreins' multiple forms were 4.2—4.5 (dog), 4.1 (rat), and 3.6—4.2 (hog).

Dog pancreatic kallikrein was not inhibited by Trasylol, but were inhibited considerably by two kallikrein inhibitors from potatoes. Although rat kallikrein has been reported not to be inhibited by Trasylol, our purified one was strongly inhibited. Trypsin inhibitors from soybean etc. hardly inhibited both kallikreins.

Purification or isolation of kallikreins from glandular tissues has been reported by many investigators.[2-5] In order to isolate the kallikreins, however, many purification steps were involved, so that the yield of isolated kallikrein decreased. Most glandular kallikreins were reported to be acidic proteins of isoelectric points 3—4.5 and molecular weights 24000—40500.[2-5] Also it has been observed that their electrophoretic migrations in disc electrophoresis at pH 8—9 are very rapid. Consideration of these properties of the glandular kallikreins, led us to attempt their purification mainly by preparative disc electrophoresis.

This paper deals with the purification process for dog, rat and hog pancreatic kallikreins, combining preparative disc electrophoresis with acetone fractionation and Sephadex gel filtration. It also deals with some properties of the first two kallikreins, about whose purification and properties little has been reported. The third one is important since it is the most typical glandular kallikrein.

Material and Method

Kallikreins and Proteinase Inhibitors——The pancreas excised from rats and dogs were stored at −20°, and used after removal of fats from the tissues. A partially purified preparation of hog pancreatic kallikrein (133 KU/mg, 10.5 BAEE μmoles/min/mg) was obtained by the acrinol method of Moriya.[6]

1) Location: *12, Ichigaya-Funagawara-machi, Shinjuku-ku, Tokyo.*
2) *a*) E.K. Frey, H. Kraut and E. Werle, "Das Kallikrein-Kinin System und seine Inhibitoren," Ferdinand Enke Verlag, Stuttgart, 1968, p. 18; *b*) J.V. Pierce, "Handbook of Experimental Pharmacology," Vol. XXV, ed. by E.G. Erdös, Springer-Verlag, Berlin, 1970, p. 21; *c*) M.E. Webster, *ibid.*, p. 131.
3) H. Moriya, A. Kato and H. Fukushima, *Biochem. Pharmacol.*, **18**, 549 (1969); Y. Fujimoto, H. Moriya and C. Moriwaki, *J. Biochem.* (Tokyo), **74**, 239 (1973); C. Moriwaki, N. Watanuki, Y. Fujimoto and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), **22**, 628 (1974).
4) *a*) H. Fritz, I. Eckert and E. Werle, *Z. Physiol. Chem.*, **348**, 1120 (1967); *b*) F. Fiedler, C. Hirschauer and E. Werle, *ibid.*, **351**, 225 (1970); *c*) F. Fiedler, B. Müller and E. Werle, *ibid.*, **351**, 1002 (1970).
5) *a*) E. Habermann, *Z. Physiol. Chem.*, **328**, 15 (1962); *b*) T. Takami, *Seikagaku*, **41**, 777 (1969); *c*) C. Kutzbach and G. Schmidt-Kastner, *Z. Physiol. Chem.*, **353**, 1099 (1972).
6) H. Moriya, *Yakugaku Zasshi*, **79**, 32 (1959).

Two kallikrein inhibitors from potatoes (PKI-56 and -64) were prepared in our laboratory.[7] Trasylol (commercially available kallikrein–trypsin inhibitor from bovine organs)[8] was presented by Bayer AG, Germany, and soybean,- lima bean-, and egg white-trypsin inhibitors [SBTI (type I-S), LBTI (II-L), and EWTI (II-O) respectively] were purchased from Sigma Chem. Co. Heparin (160 units/mg) was a product of Taiyo Fishery Co., Ltd., Tokyo. Other chemicals were the same as used in the previous paper.[9]

Preparative Disc Electrophoresis——The Prep-Disc equipment and the chemicals were products of Canal Industrial Corporation. The procedure was done according to the instruction manual of the Corporation, which is based on the disc electrophoresis method of Davis.[10] The detailed for electrophoresis operations are described in the Result.

Vasodilator Activity——Determination of this activity of kallikrein was performed by the increase of blood flow of the femoral artery of the dog, as previously described.[9,11]

Esterolytic Activity——This was determined at 25° and pH 8.0 (in the absence of calcium ions) by the same spectrophotometric methods[12] as in the previous report,[9] using a Hitachi spectrophotometer, model 124. Synthetic substrates used were N^{α}-benzoyl-L-arginine ethyl ester (BAEE, at 253 nm),[12a] N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester (TAME, at 247 nm),[12b] N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA, at 405 nm),[12c] and N-benzoyl-L-tyrosine ethyl ester (BTEE, at 256 nm).[12b]

Caseinolytic Activity——Caseinolysis was determined at 35° by the method of Kunitz.[13]

Determination of Inhibitor Activity——The inhibitory effects of proteinase inhibitors on dog and rat pancreatic kallikreins were examined as follows. Equal volumes of kallikrein (constant concentration) and each inhibitor (2—6 graded concentrations) or 0.05M Tris-HCl (pH 8.0) for the control were mixed and preincubated for 30 min at 37°. All of the kallikreins and the inhibitors had been dissolved in the same buffer. Then the remaining kallikrein activity was determined by the dog vasodilator and BAEE esterolytic assay methods as previously described.[7]

Kallikrein and Inhibitor Units——The activity of kallikreins was given by kallikrein unit (KU) in the vasodilator assay, and by μmole of synthetic substrate hydrolyzed in the esterolytic assay. Caseinolysis was denoted by Kunitz's unit, $[TU]^{cas.}_{mg}$. Inhibitory activity of Trasylol or PKI's was sometimes expressed by kallikrein inhibitor unit (KIU).[14] One KIU is the amount of inhibitor needed to inhibit 1 KU in the dog assay.

Disc Electrophoresis and Other Experiments——Disc electrophoresis,[10] Ampholine isoelectric focusing,[15] and gel isoelectrofocusing[9] were carried out under the almost same methods and experimental conditions described in the previous paper.[9]

Result

Isolation of Dog Pancreatic Kallikrein (Dog Panc. K.)

Step 1——The pancreas from mongrel dogs, 18.3 g, were minced and homogenized with 10 ml of deionized water. Then 82 ml of water were further added to the homogenate and stirred for about 8 hr at 20°. The homogenate was centrifuged, and a supernatant of 96 ml was obtained.

Step 2——The supernatant was diluted twice with water, adjusted from pH 6.0 to 7.0 with 5N NaOH, and fractionated with acetone at below 10°. Precipitates formed at 50% (v/v) concentration of acetone were centrifuged off, and those formed between 50—67% were collected and suspended in 3.2 ml of water. Insoluble materials were removed by centrifugation.

7) Y. Hojima, C. Moriwaki and H. Moriya, *J. Biochem.* (Tokyo), **73**, 923 (1973); *idem, ibid.*, **73**, 933 (1973).
8) I. Trautschold, E. Werle and G. Zickgraf-Rüdel, *Arzneim.-Forsch.*, **16**, 1507 (1966).
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10) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
11) C. Moriwaki, Y. Hojima and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), **22**, 975 (1974).
12) *a*) G.W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, **16**, 570 (1955); *b*) B.C.W. Hummel, *Can. J. Biochem. Biophys.*, **37**, 1393 (1959); *c*) I. Trautschold, "Handbook of Experimental Pharmacology," Vol. XXV, ed. by E.G. Erdös, Springer-Verlag, Berlin, 1970, p. 52.
13) M. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).
14) R. Vogel and E. Werle, "Handbook of Experimental Pharmacology," Vol. XXV, ed. by E.G. Erdös, Springer-Verlag, Berlin, 1970, p. 213.
15) O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, **20**, 820 (1966).

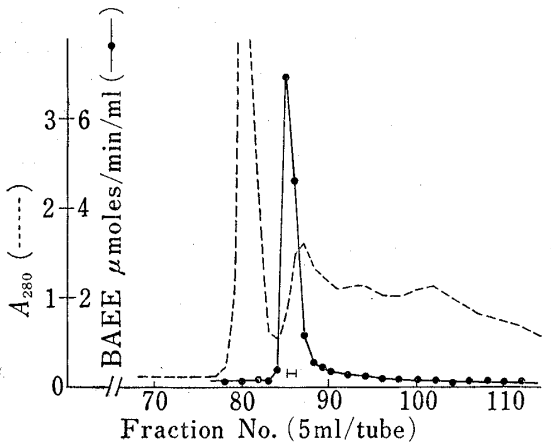

Fig. 1. Preparative Disc Electrophoresis of Dog Panc. K. (Step 3)

The recoveries of protein and activity were nearly 100% in this electrophoresis. Bracketed fractions were combined and used in step 4.

Step 3——A PD2/320 column was used as an upper column in the following preparative electrophoresis. Its volume was 3.5 ml per cm of gel height. The height of the separation gel [7% (w/v) polyacrylamide] which was placed in the lower part of the column, and that of a spacer gel [3.5% (w/v)] were 6 and 3 cm respectively. The supernatant (3.8 ml) which was obtained in step 2 was mixed with 1.9 ml of 0.49 M Tris-HCl [pH 6.7, contained 0.46 ml of N, N, N′,N′-tetramethylethylenediamine(TEMED) per 100 ml] and 0.95 ml of 50% (w/v) sucrose. This mixture was laid on the spacer gel. As an electrode solution 0.025 M Tris–0.19 M glycine (pH 8.3) was used. Electrophoresis was done at 250 V, cooling the column below 20° by running tap water. Elution was performed with 0.1 M Tris-HCl (pH 8.3) at a flow rate of about 50 ml/hr. Protein concentration and BAEE hydrolysis of the effluent collected are shown in Fig. 1. Fractions 85 and 86 in Fig. 1 were combined and used in the next step.

Step 4——The combined fractions were filtered through a column of Sephadex G-75 (2.5×94 cm) equilibrated with deionized water at 4° with a flow rate of about 20 ml/hr. Two fractions with the highest specific activity were combined and lyophilized. The preparation (1.4 mg) was homogeneous in analytical disc electrophoresis (Fig. 2). Its enzymatic activities were 867 KU/mg in vasodilator assay and 18.3 μmoles/min/mg in BAEE assay. Specific activities, amounts of total proteins, *etc.* of the respective purification steps are summarized in Table I. Through the overall purification procedures, the recoveries of the vasodilator and of the esterolytic activities were 28 and 36% respectively.

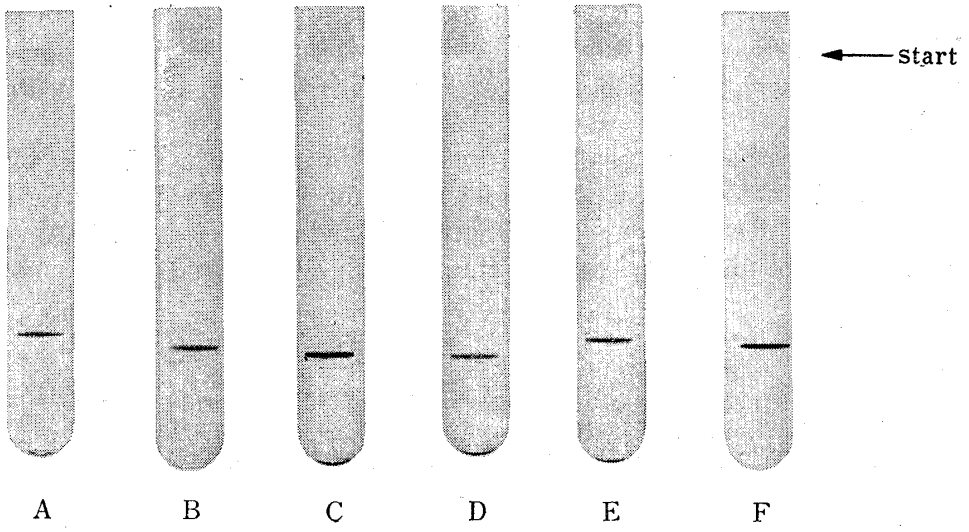

Fig. 2. Disc Electrophoresis of Dog, Rat and Hog Pancreatic Kallikreins

A: Dog Panc. K. (14 μg), B: sample B of Rat Panc. K. (0.03 A_{280}), C: Rat Panc. K. isolated from sample B (0.04 A_{280}), D: sample C of Rat Panc. K. (0.03 A_{280}), E: Rat Panc. K. isolated from sample C (0.03 A_{280}), F: Hog Panc. K. (20 μg)

electrophoresis[19]: 7% (w/v) polyacrylamide gel, 2mA per tube (0.5×8.5 cm), pH 8.9, 1.5 hr, 4°

Stained with 0.01% (w/v) coomassie brilliant blue dissolved in 10% (w/v) trichloroacetic acid.

Table I. Summary of Purification of Dog Panc. K.

Step	Procedure	Total proteins (A_{280})	Vasodilator act.		BAEE hydrolysis	
			Total KU	KU/A_{280}	Total units[a]	Units/A_{280}
1	extraction	7 680	4 390	0.57	71.0	0.0093
2	acetone fractionation [50—67 % (v/v)]	331	4 230	12.8	70.7	0.21
3	preparative disc electrophoresis	9.8	2 890	296	45.9	4.71
4	gel filtration with Sephadex G-75	1.4[b]	1 210	867[c]	25.6	18.3[d]

a) unit: μmole/min *b*) mg *c*) KU/mg *d*) units/mg

Ampholine Isoelectric Focusing of Dog Panc. K.

As shown in Fig. 3, several activity peaks of Dog Panc. K. were found and their isoelectric points (pI's) were 4.22, 4.30, 4.40 and 4.53. Therefore, although this preparation of Dog Panc. K. gave a single protein band in disc electrophoresis (Fig. 2), it appears to consist of several kallikrein multiple forms with slightly different pI's just as in the cases of hog pancreatic and human urinary kallikreins.[9] We discussed this problem in the preceding paper.[9] (The four peaks in Fig. 3 showed the vasodilator activity of 1—5 KU/ml proportional to their esterolytic activities).

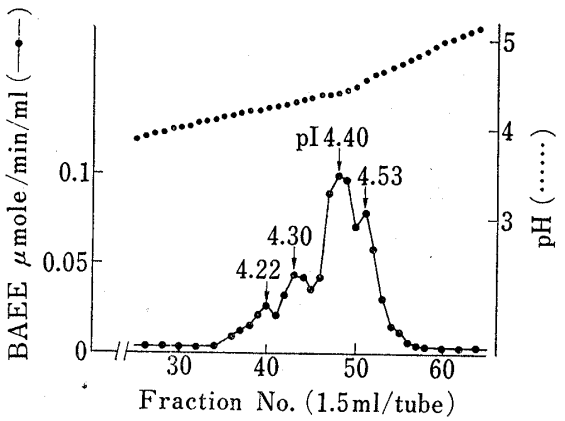

Fig. 3. Ampholine Isoelectric Focusing of Dog Panc. K.

Dog Panc. K.: 0.085 mg (867 KU/mg, 18.3 BAEE μmoles/min/mg), column: 110 ml, Ampholine: pH 3—5, 1% (w/v), focusing: 500 V, 35 hr, 4°
The recovery of the activity after focusing was 78%.

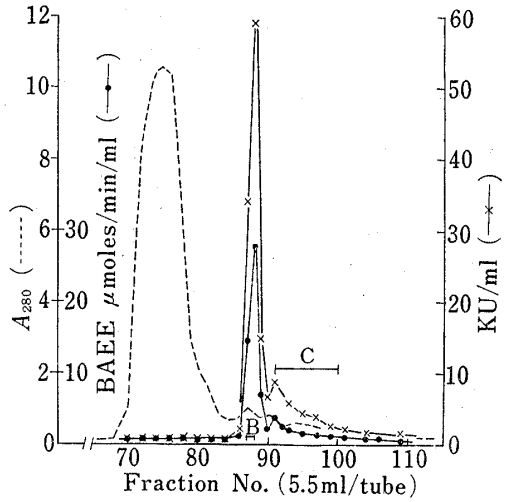

Fig. 4. Preparative Disc Electrophoresis of Rat Panc. K. (Step 3)

The recoveries of protein and esterolytic and vasodilator activities after electrophoresis were 96, 47 and 56% respectively. Two groups of bracketed fractions (B and C) were pooled separately and used in step 4.

Isolation of Rat Pancreatic Kallikrein (Rat Panc. K.)

Step 1——The pancreas of rats (Donryu strain), 18.8 g, were minced and homogenized with 30 ml of water. Afterward, 160 ml of water were added into the homogenate and stirred for about 2 hr at 25°. The pH of the homogenate was adjusted from 6.2 to 7.2 with 5N NaOH, and then the homogenate was centrifuged. Two hundred milliliters of the supernatant were obtained.

Step 2——Acetone was added dropwise to the above extract in an ice bath to 50% (v/v) concentration, and 20 min later the precipitates formed were removed by centrifugation. Then acetone was added to the supernatant to 67%, and the precipitates were collected and dissolved

in 28 ml of 0.3M NaCl. This solution was again fractionated with acetone in the same manner. Precipitates formed between 52—67% (v/v) were collected and dissolved in 6 ml of deionized water. This highly active fraction (6.7 ml) was named sample A.

Step 3——A large portion of sample A, 5.8 ml (equivalent to 16.3 g of the starting material), was submitted to preparative disc electrophoresis. The PD2/320 column was also used as an upper column. The heights of separation gel [7% (w/v) polyacrylamide] and spacer gel (3.5%) were 5.8 and 3 cm respectively. The sample was layered on the spacer gel after mixed with 5.8 ml of 0.49M Tris-HCl (pH 6.7, contained 0.46 ml of TEMED per 100 ml), 2.9 ml of 50% (w/v) sucrose and 5.8 ml of water (5.8 cm in height). The electrode solution was 0.025M Tris–0.19M glycine (pH 8.3). Electrophoresis was performed at 350 V, and the flow rate of the eluant (0.1M Tris-HCl, pH 8.0) was 75 ml/hr (Fig. 4).

Fractions 87 and 88 (Fig. 4) were combined and named sample B. Most of sample B (7.8 ml) was applied to the next gel filtration step (step 4). Fractions between No. 91—100 (Fig. 4) were pooled and concentrated to 11.6 ml by an Amicon ultrafiltration cell (model 402) with a UM-20E membrane (Amicon Corp.). This solution was named sample C, and also gel-filtered as described in the next step.

Step 4——Sample B (7.8 ml, equivalent to 11.5 g of the starting material) was filtered through a Sephadex G-100 column (2.5×94 cm) equilibrated with deionized water at 4°. Two fractions with the highest specific activity were combined. This preparation (purified from sample B) had activities of 301 KU/A_{280} and 124 BAEE μmoles/min/A_{280}, and its recoveries of the vasodilator and esterolytic activities were 24 and 22% respectively (Table II). This purified Rat Panc. K. was homogeneous in disc electrophoresis (Fig. 2). (The sample C was also purified homogeneously by the Sephadex G-100 column, as shown in Fig. 2).

Table II. Summary of Purification of Rat Panc. K.[a]

Step	Procedure	Total proteins (A_{280})	Vasodilator act.		BAEE hydrolysis	
			Total KU	KU/A_{280}	Total units[b]	Units/A_{280}
1	extraction	10 050	1 090	0.11	493	0.049
2	second fractionation (acetone concn.: 52—67%)	420	1 006	2.41	609	1.45
3	Prep. disc electrophoresis (No. 87—88, sample B)	7.5	363	48.5	164	21.9
4	gel filtration with Sephadex G-100	0.85	259	301	107	124

a) This table has been summarized assuming that 11.5 g of the rat pancreas were used. See step 4 in the text.
b) unit: μmole/min

Isoelectric Focusings of Three Samples (A, B and C) of Rat Panc. K.

All pI's of the main activity peaks of samples A—C were about 4.1 (Fig. 5), but these peaks did not seem symmetrical. Samples B and C of step 3 were purified almost homogeneously as shown in Fig. 2. However, in gel isoelectrofocusing they were divided into 4—5 protein bands (Fig. 6). Further the molecular weights of Rat Panc. K.'s of both samples B and C have been estimated to be 30000 by the gel filtration technique with Sephadex G-100.[16] From these data, it was considered that the Rat Panc. K.'s contained in these samples were the same and were made up of several kallikrein multiple forms. Why two fractions of Rat Panc. K.'s were eluted being separated in the preparative electrophoresis still remains as an undissolved question. (The vasodilator activity of the peak around pI 4.8 in Fig. 5A was negligible. Judging from its pI, the enzyme of this peak may be an anionic trypsin.[17])

16) J.R. Whitaker, *Anal. Chem.*, **35**, 1950 (1963).
17) Y. Hojima, M. Yamashita and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), **23**, 225 (1975).

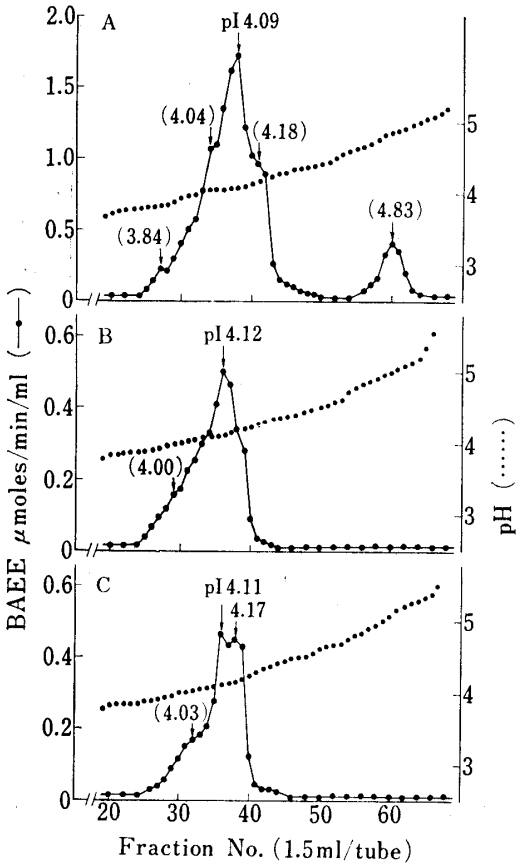

Fig. 5. Isoelectric Focusings of Samples A, B and C of Rat Panc. K.

A: sample A, 41.0 A_{280} (2.41 KU/A_{280}, 1.45 BAEE μmoles/min/A_{280})
B: sample B, 0.77 A_{280} (48.5 KU/A_{280}, 21.9 BAEE μmoles/min/A_{280})
C: sample C, 1.06 A_{280} (10.0 KU/A_{280}, 6.23 BAEE μmoles/min/A_{280})
column: 110 ml, Ampholine: pH 3—5, 1% (w/v), focusing: 500 V, 37—41 hr, 4°
The recoveries of the activities of samples A, B and C after focusing were 43, 35 and 71% respectively.

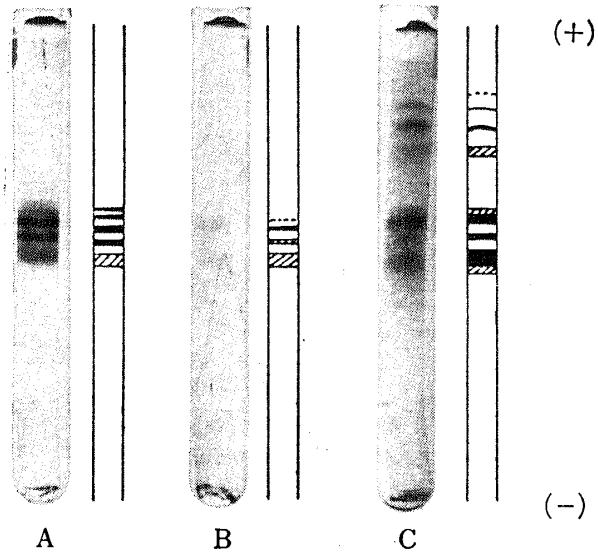

Fig. 6. Gel Isoelectrofocusing of Rat and Hog Pancreatic Kallikreins

A: sample B of Rat Panc. K. (0.1 A_{280})
B: sample C of Rat Panc. K. (0.05 A_{280})
C: Hog Panc. K. (75 μg)
gel: 7% (w/v) polyacrylamide gel (0.5×8.5 cm) containing 1% (w/v) Ampholine with a pH range of 3—5, isoelectrofocusing[9]: 250 V, 6.5 hr, 4°, anode: 0.2% (v/v) phosphoric acid, cathode: 0.4% (v/v) ethylenediamine.
Gels were washed for 24 hr with 10% (w/v) trichloroacetic acid and stained with coomassie brilliant blue.

Isolation of Hog Pancreatic Kallikrein (Hog Panc. K.)

Step 1——Fifty-six milligrams of partially purified Hog Panc. K. (133 KU/mg, 10.5 BAEE μmoles/min/mg) were dissolved in 1.6 ml of deionized water.

Step 2——Into the above solution, 0.8 ml of 14% (w/v) acrylamide (contained 250 mg of N, N′-methylenebisacrylamide per 100 ml), 0.4 ml of 0.49M Tris-HCl (pH 6.7), 0.4 ml of riboflavine (0.04 mg/ml) and 2 drops of bromphenol blue [0.005% (w/v)] were added, and this mixture was gellified on a spacer gel. As an upper column, a PD2/70 column (1 ml per cm of gel height) was used. The heights of separation gel [7% (w/v) polyacrylamide], spacer gel (3.5%) and sample gel (3.5%) were 6, 3.5 and 3.2 cm respectively. Electrophoresis was done at 200 V, and 0.15M Tris-HCl (pH 8.0) was used as eluant at a flow rate of about 50 ml/hr. Fractions 55—60 in Fig. 7 were pooled.

Step 3——The above pooled solution was gel-filtered through a Sephadex G-50 column (2.5×93 cm, equilibrated with water). A break-through peak in this filtration was lyophilized. The preparation obtained (2.7 mg) was homogeneous in disc electrophoresis (Fig. 2), and showed the activities of 1170 KU/mg and 89.5 BAEE μmoles/min/mg. The overall recoveries of both activities through the above steps were 42 and 41% respectively (Table III).

As shown in Figs. 6 and 8, gel isoelectrofocusing and isoelectric focusing separated the above preparation into several protein bands and into two main and two subsidiary activity peaks respectively. These were very similar to those obtained for Hog Panc. K. described in the previous paper.[9]

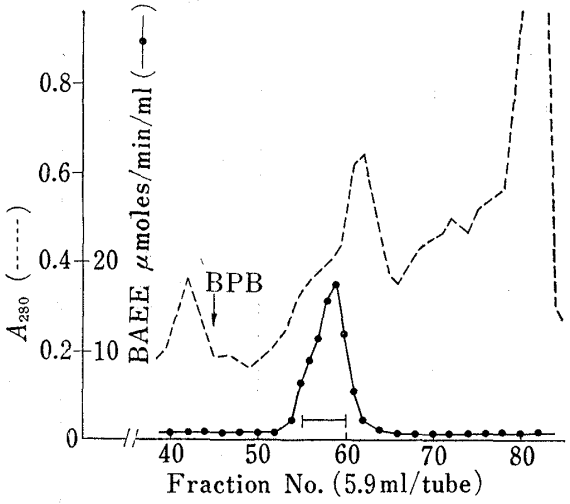

Fig. 7. Preparative Disc Electrophoresis of Hog Panc. K. (Step 2)

The recoveries of protein and activity after electrophoresis were 139 and 78% respectively. Bracketed fractions were pooled and used in step 3. BPB: bromphenol blue

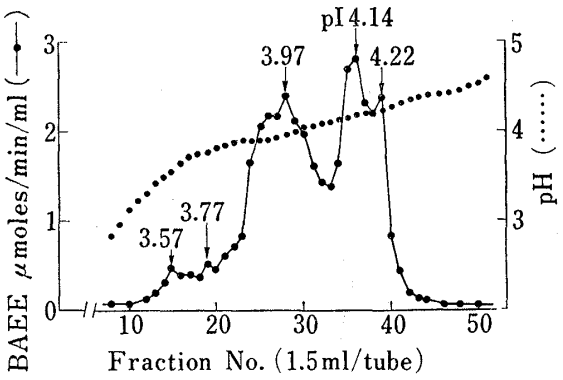

Fig. 8. Isoelectric Focusing of Hog Panc. K.

Hog Panc. K.: 0.71 mg (1 170 KU/mg, 89.5 BAEE μmoles/min/mg), column: 110 ml, Ampholine: pH 3—5, 1% (w/v), focusing: 500 V, 40 hr, 4°
The recovery of the activity after focusing was 94%.

TABLE III. Summary of Purification of Hog Panc. K.

Step	Procedure	Total proteins (mg)	Vasodilator act.		BAEE hydrolysis	
			Total KU	KU/mg	Total units[a]	Units/mg
1	partially purified Hog Panc. K.	56.0	7 450	133	588	10.5
2	preparative disc electrophoresis	12.6[b]	—	—	380	30.0[c]
3	gel filtration with Sephadex G-50	2.7	3 160	1 170	242	89.5

a) unit: μmole/min *b*) A_{280} *c*) units/A_{280}

Effects of Proteinase Inhibitors on Dog and Rat Panc. K.'s

Inhibitory effects of proteinase inhibitors against both kallikreins are shown in Table IV. Trasylol, although not inhibitory against Dog Panc. K. as reported,[18] was strongly inhibitory toward Rat Panc. K. Assuming 1 KIU of Trasylol to be equivalent to 0.14—0.15 μg,[8] its potency against Rat Panc. K. in the dog assay was calculated from 55% inhibition by 12.5 KIU to be 3300 KIU/mg. On the other hand, PKI-56 and -64 considerably inhibited Dog Panc. K. From the 37 and 36% inhibitions of PKI-56 and -64 by 25 μg, 220 and 210 KIU/mg were calculated as their respective inhibitory activities. The inhibitory effect of SBTI was weak (Table IV), and those of LBTI and EWTI were negligible. Heparin, which has been reported to inhibit dog plasma kallikrein,[19] was not inhibitory against Dog Panc. K. in the range of 5—500 units/ml of preincubation mixture.

Substrate Specificities and Some Properties

Enzymatic activities on several substrates and extinction coefficients of three isolated kallikreins are summarized in Table V. The ratios of KU/mg to BAEE μmoles/min/mg of Dog, Rat and Hog Panc. K.'s were about 50, 2.5 and 13 respectively, and the activity

18) R. Vogel, *Z. Physiol. Chem.*, **349**, 926 (1968).
19) N. Back and R. Steger, *Proc. Soc. Exptl. Biol. Med.*, **133**, 740 (1970).

TABLE IV. Inhibitory Effects of Proteinase Inhibitors on Dog and Rat Panc. K.'s

Inhibitor (KIU or μg/ml of preincub. mix.)		Inhibition (%)			
		Dog Panc. K.[a]		Rat Panc. K.[b]	
		Dog assay	BAEE assay	Dog assay	BAEE assay
Trasylol	6.3 KIU	—	—	34	19
	12.5	—	—	55	39
	25	—	—	86	82
	50	—	—	93	94
	100	11	9	97	96
	200	—	—	97	98
	500	19	22	—	—
	2 500	43	59	—	—
SBTI	50 μg	—	—	10	12
	100	3	0	16	13
	500	12	0	54	26
PKI-56	12.5 μg	21	34	—	—
	25	37	48	—	—
	50	48	58	—	—
	100	84	83	27	29
	500	96	94	52	46
PKI-64	12.5 μg	20	43	—	—
	25	36	58	—	—
	50	62	68	—	—
	100	85	87	40	34
	500	98	94	66	55

a) Partially purified Dog Panc. K. (394 KU/mg, 8.9 BAEE μmoles/ min/mg) was used. This was prepared by lyophilization of the dialysate of the active fractions obtained in a different preparative disc electrophoresis. The activities of the control tube (preincubated) were 14.7 KU/ml and 0.31 BAEE μmole/min/ml.

b) A homogeneous, lyophilized preparation of Rat Panc. K. (806 KU/mg, 300 BAEE μmoles/min/mg, prepared in a different purification) was used. The activities of the control were 10.8 KU/ml and 4.5 BAEE μmoles/ min/ml.

ratios of BAEE to TAME of those kallikreins were about 2, 7 and 15 respectively. From the previous results for various glandular kallikreins,[9,11] it seemed to be characteristic that Dog Panc. K. hydrolyzed BAEE 2 times more rapidly than TAME. Furthermore, it was very noticeable that Rat Panc. K. had a potent BAEE hydrolytic activity compared with its vasodilator activity. The activities against BA*p*NA, BTEE and casein were weak or not detectable.

TABLE V. Substrate Specificities and Extinction Coefficients of Three Glandular Kallikreins

Kallikrein	KU/mg	μmoles/min/mg[a]				[TU] cas. mg	$E\,\frac{1\,\%}{1\ \mathrm{cm}}$[b]
		BAEE	TAME	BA*p*NA	BTEE		
Dog Panc. K.	867	18.3	10.0	N D[c]	0.05	N D	11.5
Rat Panc. K.[d]	301	124	18.9	0.061	0.06	0.04	20.3[e]
Hog Panc. K.	1 170	89.5	5.9	0.006	N D	N D	15.8

a) determined at 25°, pH 8.0 in the absence of calcium ions

b) extinction coefficient at 280 nm, in 0.05M Tris-HCl (pH 8.0)

c) not detectable

d) Enzyme activities of this kallikrein are expressed with the values per A_{280}.

e) Determined by the homogeneous preparation with the activities of 806 KU/mg and 300 BAEE μmoles/ min/mg, which was obtained in a different purification.

The extinction coefficient of Rat Panc. K. was larger than those of Dog and Hog Panc. K.'s. That of Hog Panc. K. was in good agreement with those of other investigators.[4a,5c] The molecular weights of Dog, Rat and Hog Panc. K.'s were estimated to be 28000, 30000 and 31000 respectively by gel filtration with Sephadex G-100.[16] The first two kallikreins were quite stable at pH 4—10 and 25° for 24 hr, more than 90% of the activity remaining after the treatment.

Discussion

Kallikrein contents in the dog and rat pancreas have been reported to be 10—100 KU and 70—80 KU per gram respectively.[2c] The values obtained in our studies, 50—250 KU (mongrel dogs) and 60—250 KU (Donryu and Wistar strain rats), agree with or are slightly larger than the above reported ones. For the other rodents such as mouse (DD strain) and guinea-pig (Hartley strain), only about 15 KU and negligible amounts per gram of the pancreas respectively were observed.

From the results shown in Tables I and II, preparative disc electrophoresis appears to be a preferable method for the purification of glandular kallikreins which are acidic proteins. Dog and Rat Panc. K.'s were purified 15- to 25-fold by this procedure with the activity recoveries of 30—60% from the preceding step. This procedure was also applicable for the preparation of other highly purified glandular kallikreins with good recoveries, for instance, human urinary and human salivary kallikreins. The former with 74 KU/mg (from 5 KU/mg) and the latter with 90 KU/mg (from 2 KU/mg) were obtained by combining such procedures with Sephadex gel filtration as described in this paper.

From the results presented in the above figures, the following conclusion may be induced: Dog and Rat Panc. K.'s as well as Hog Panc. K. are composed of kallikrein multiple forms with slightly different pI's. Particularly, Rat Panc. K. seemed to consist of multiplicity of forms. The results from isoelectric focusing, gel isoelectrofocusing and disc electrophoresis support our conclusion regarding the homogeneity of glandular kallikrein.[9]

Trasylol has been reported not to be inhibitory against Dog and Rat Panc. K.'s.[18] We obtained the same results on Dog Panc. K. as reported, but highly purified Rat Panc. K. was inhibited by Trasylol (Table IV). This discrepancy may be ascribed to the purity of kallikrein preparations used. Crude Rat Panc. K. (2.1 KU/A_{280}, 1.4 BAEE μmoles/min/A_{280}) was likewise inhibited by Trasylol in our assay, but more weakly than the purified one. Namely, about 400 KIU/mg was obtained as the inhibitory activity of Trasylol, whose activity was smaller than that against purified Rat Panc. K. (3300 KIU/mg, Table IV). Thus, since the preparation which Werle's group used probably was a crude one, it would have been difficult for them to detect the weak inhibition of Rat Panc. K. by Trasylol. The weak inhibitory activity of Trasylol against a crude preparation of Rat Panc. K. may be due to the higher affinity for the contaminating proteinases (like trypsin) than for Rat Panc. K. Meanwhile, though the inhibitory activities of PKI's against Rat Panc. K. were very weak, those against Dog Panc. K. were considerably strong; and this seemed to be worth noting.