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A Proposal—Use of Combined Assays of Kallikrein Activity Measurement

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Though there are several assay methods for the kallikreins, there are neither standard units nor standard methods. The representative activities of kallikreins, such as the vasodilator, kinin forming and esterolytic activities, were determined by various methods on hog pancreatic, human salivary, urinary and plasma and guinea pig coagulating gland kallikreins and also on trypsin. Some enzymatic properties of these kallikreins were discussed from comparison of their activities, and the procedures of these assay methods were also examined in detail.

Since the discovery of kallikreins in urine, pancreas and other tissues many investigators have studied their purification, mode of action and physiological and pathological significance. In these investigations the method of assay is an important problem as the components involved in the kallikrein–kinin system are rapidly metabolized and their contents in tissue are scarce, extensively variable and dependent upon the physical condition of the animal and its species.

The hypotensive action of the kallikreins has been of great use in determining their activity, but measurement of blood flow has taken its place because of increased sensitivity and other advantages.[2] Isolated smooth muscle tissues, such as guinea pig ileum or rat uterus, are contracted by the kinins, and this response has also been applied as an assay method. Meanwhile, Webster and Pierce[3] found that kallikreins hydrolyze N-substituted arginine esters and that this esterolytic activity was nearly proportional to the vasodilator activity, especially in human urine.

These three methods are the usual assay methods for the kallikreins and kinins. However, there are neither unified units, standard methods nor detailed comparisons of the potencies determined by these methods, making it difficult to compare experimental results with other investigators. To clarify this situation, a meeting held in 1963 in Florence, Italy, made recommendations for the nomenclature and for the units of this enzyme system which have been compiled by Webster.[4] The present paper represents details of the typical assay results of different kinds of kallikreins, and the authors would like to propose use of com-

1) Location: *12, Ichigaya-Funagawara-machi, Shinjuku-ku, Tokyo.*
2) H. Moriya, K. Yamazaki, and H. Fukushima, *J. Biochem.* (Tokyo), **58**, 201 (1965).
3) M.E. Webster and J.V. Pierce, *Proc. Soc. Exptl. Biol. Med.*, **107**, 186 (1961).
4) *a*) M.E. Webster, "Hypotensive Peptides," ed. by E.G. Erdös, N. Back and F. Sicuteri, Springer-Verlag, New York, 1966, p. 648; *b*) *Idem*, "Handbook of Experimental Pharmacology, Vol. XXV, Bradykinin, Kallidin and Kallikrein," ed. by E.G. Erdös, Springer-Verlag, Berlin, 1970, p. 659.

bined units for the activity of kallikreins of different sources and purifications, in comparison of various activities of kallikreins, such as hypotensive, vasodilator, kinin forming, and esterolytic activities. Furthermore, some properties of these kallikreins were discussed by comparison of their activities.

Material and Method

Kallikreins——Partially purified preparations of hog pancreatic kallikreins (KZ-6, KY-3, KU-9, and 701-F in Table I) were obtained by the method of Moriya, *et al.*[5] Highly purified kallikrein KK-2 was prepared as described previously[6] and KZC-150[7] (1250 KU/mg) was presented from Bayer Pharmaceutical Co., Germany. A commercial kallikrein preparation, Padutin (10 biological units per ampoule), was also presented from Bayer Co. Human salivary kallikrein was partially purified with slight modification of the previous method.[2] Crude human plasma kallikrein was prepared by acetone precipitation (75% v/v) from acetone activated human plasma.[8] Crude human urinary kallikrein, ammonium sulfate precipitate of a silica-gel adsorbed fraction of urine,[9] was obtained from Green Cross Corp., Osaka, and was purified by Moriya, *et al.*[10] Kallikrein of the coagulating gland of the guinea pig was extracted with water and partially purified by Moriwaki and Schachter.[11] Twice crystallized bovine trypsin was purchased from Sigma Chemical Co., U.S.A.

Hypotensive and Vasodilator Assay——Normal adult dogs, weighing 7—12 kg, were used for these assays. The arterial blood pressure and blood flow were measured at the common carotid and the femoral arteries, respectively, under pentobarbital anesthesia. The assay samples dissolved in 0.2—0.4 ml of physiological saline solution were injected through the femoral vein in depressor assay or through the femoral artery in vasodilator assay, and the responses were measured by an electronic manometer (MP-3A, Nihon-Koden, Tokyo) or an electromagnetic flowmeter (MF-2) respectively. Sample administration was performed every 15 min in the former assay, and 4 min in the latter. The standard preparation for these assays was the hog pancreatic kallikrein prepared in our laboratory (701-F). Its vasodilator activity was determined as 23.25 KU/mg when compared to that of the standard kallikrein (hog pancreatic), courteously supplied by Dr. M.E. Webster, National Institutes of Health, U.S.A., and this activity was confirmed by Dr. E. Werle, München University, Germany. Both the hypotensive and the vasodilator activities were given in kallikrein unit (KU).[4,12]

Kinin Forming Assay——The samples were incubated with 15 mg of bovine kininogen fraction which was prepared from heated plasma (60° for 1 hr) by precipitation with a half saturation of ammonium sulfate, dialysis against distilled water for 2 days with several changes, and lyophilization. One milligram of this kininogen yielded 100 ng of bradykinin by an excess amount of trypsin and it was confirmed to be free from plasma kallikrein, kininases and kinins. The incubation of sample and kininogen was usually carried out at 30° for 1 min in 0.02 M phosphate buffer (pH 7.5), and the liberated kinin was estimated by its smooth muscle contractive activity.

An isolated guinea pig ileum segment was suspended in a 10 ml bath of air saturated Mg^{2+}-free Tyrode's solution (contained 2×10^{-2} mg each of atropine and mepyramine in 1 liter). Under 4 g of tension the contractile response with 0.1—0.5 ml of the incubated mixture of sample and kininogen was measured isometrically with a Force-Displacement Transducer (SB-1T, Nihon-Koden, Tokyo). Synthetic bradykinin (BRS 640, Sandoz Pharmaceuticals, Switzerland) was used as the standard in this assay system, and the kinin forming activity was expressed in terms of μg of bradykinin equivalent.

Silicone coated glass-wares were used throughout the preparation of kininogen and during the kinin forming reaction.

Esterolytic Assay——N-α-Tosyl-L-arginine methyl ester (TAME) and N-α-benzoyl-L-arginine ethyl ester (BAEE), purchased from Protein Research Foundation, Mino, Japan, were used as substrates, and the following methods were employed for the determination of the esterolytic activity of the enzyme samples.

According to Webster and Pierce[3] 1.0 ml of 0.06 M TAME aqueous solution, 1.0 ml of 0.75 M Tris-HCl buffer (pH 8.5) and 1.0 ml of the sample solution were mixed and incubated at 30° for 1 hr. An 1.0 ml aliquot of the incubated was added to a mixture of 1.0 ml each of 2.0 M $NH_2OH \cdot HCl$ and 3.5 M NaOH, and was kept at 24° for 25 min. The formation of hydroxamic acid from the residual TAME was stopped by addition

5) H. Moriya, K. Yamazaki, H. Fukushima, and C. Moriwaki, *J. Biochem.* (Tokyo), **58**, 208 (1965).
6) H. Moriya, A. Kato, and H. Fukushima, *Biochem. Pharmacol.*, **18**, 549 (1969).
7) C. Kutzbach and G. Schmidt-Kastner, *Z. Physiol. Chem.*, **353**, 1099 (1972).
8) H. Moriya, K. Yamazaki, and H. Fukushima, *J. Biochem.* (Tokyo), **58**, 315 (1965).
9) E. Sako, Y. Mima, and O. Kawamura, Japan Patent 19067 (1971).
10) H. Moriya, J.V. Pierce, and M.E. Webster, *Ann. N. Y. Acad. Sci.*, **104**, 172 (1963).
11) C. Moriwaki and M. Schachter, *J. Physiol.* (London), **219**, 341 (1971).
12) E.K. Frey, H. Kraut, and E. Werle, "Kallikrein (Padutin)," F. Enke Verlag, Stuttgart, 1950.

of 1.0 ml of 0.38 M TCA—4M HCl. After removal of the insoluble precipitate by filtration, 1.0 ml of the filtrate was added to 4.0 ml of 0.11 M $FeCl_3$ and the formed ferric complex was determined colorimetrically at 525 nm. The amount of the residual substrate was estimated from the standard curve with TAME treated similarly but without enzyme, and the activity was calculated as μmoles of TAME digested in 1 min. Webster and Pierce[3] proposed an esterase unit (EU) for this activity which was defined as equal to 1 KU of human urinary kallikrein. In our experiments, EU was determined for some of the samples and, in this case, the incubation was carried out at 37°.

Recently, we developed a new assay method for this esterolytic activity by measuring the methanol liberated from TAME.[13] One tenth milliliter each of the sample solution, 0.1 M TAME aqueous solution and 0.1 M phosphate or Tris-HCl buffer (pH 8.5) were mixed and incubated at 30° for 30 min. The reaction was stopped by addition of 0.2 ml of 15% (v/v) TCA and the liberated methanol was oxidized to formaldehyde by 0.1 ml of 2% (w/v) $KMnO_4$. About 1 min later 0.1 ml of 10% (w/v) $NaHSO_3$ and 4.0 ml of freshly prepared chromotropic acid [0.4% (w/v) in 67% (v/v) H_2SO_4] were added and the solution was mixed well. The tubes were placed in a boiling water bath for 15 min, and the absorbance at 580 nm was measured after cooling to room temperature. The quantity of methanol liberated was estimated from the calibration curve obtained by 0.2 ml of 0.001—0.010 M methanol instead of the sample and TAME mixture.

The determination of the esterolytic activity on BAEE was based on the principle of Schwert and Takenaka.[14] The increase of the extinction at 253 nm (ΔE) of 3.0 ml of 10^{-3}M in 0.05 M Tris-HCl buffer (pH 8.0) was measured after the addition of 0.2 ml of sample solution at 30°, and degradation of BAEE by 1 ml of the sample solution was calculated from measured ΔE value in 1 ml with the following equation; $\Delta E/(0.359 \times 0.2)$.

Caseinolytic Assay——According to Kunitz,[15] 1.0 ml each of the sample solution and substrate solution, prepared by dissolving 1.0 g of casein (Hammarsten) in 100 ml of 0.1 M Sörensen phosphate buffer (pH 7.6), were mixed and incubated at 35° for 20 min. The reaction was stopped by addition of 3.0 ml of 5% (w/v) TCA, and the extinction of the centrifuged supernatant at 280 nm was measured. The activity was read from the initial velocity of the enzymatic reaction and expressed in Kunitz unit.

Result

Vasodilator and Hypotensive Responses

A comparison was made of the dose-response relationship of bradykinin and hog pancreatic kallikrein (109 KU/mg, KY-3) as measured by determining the increase in blood flow or the decrease in blood pressure (Fig. 1). Linearity was observed for the vasodilator response from 0.1—2 ng/kg of bradykinin and 0.1—2 mKU/kg of hog pancreatic kallikrein, whilst 5—30 ng/kg bradykinin and 10—50 mKU/kg of the kallikrein gave a linear dose-response relationship in the hypotensive response. A good parallelism was observed between the peptide and the kallikrein in both responses. Thus both methods are suitable for kallikrein assay, although the vasodilator response is about 40 times more sensitive than the hypotensive

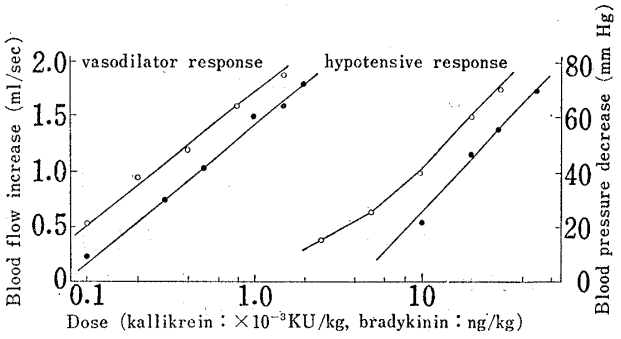

Fig. 1. Vasodilator and Hypotensive Responses in Dog by Synthetic Bradykinin and Hog Pancreatic Kallikrein

—○—: synthetic bradykinin
—●—: hog pancreatic kallikrein (KY-3, 109 KU/mg)

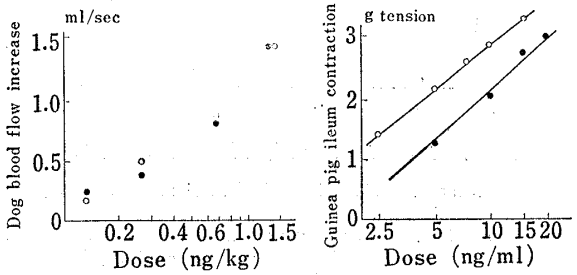

Fig. 2. Vasodilator and Smooth Muscle Contractile Responses of Synthetic Bradykinin and Kallidin

—○—: synthetic bradykinin
—●—: synthetic kallidin

13) C. Moriwaki, N. Inoue, Y. Hojima, and H. Moriya, *Yakugaku Zasshi*, **91**, 413 (1971).
14) G.W. Schwert and Y. Takenaka, *Biochem. Biophys. Acta*, **16**, 570 (1955).
15) M. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).

response. The sensitivity of the dog varied occasionally and a decrease of sensitivity was often observed during the assay.

As shown in Fig. 1, 1 μg of synthetic bradykinin corresponded to 1.8 KU hog pancreatic kallikrein in both responses, and similar results were obtained in other dogs. Furthermore, vasodilator activity of synthetic kallidin was found to be almost equal to that of synthetic bradykinin (Fig. 2), whereas its smooth muscle contractile response was the half of that of bradykinin.

In these points, Erdös has described in his review[16] that bradykinin is slightly more potent in vasodilator response than kallidin on the injection into the dog femoral artery and that bradykinin is about 3 times more potent than kallidin in guinea pig ileum contractile response. It is difficult to explain this discrepancy but there might be some differences in the experimental condition.

For determination of the kinin forming activity of the kallikrein, pseudoglobulin fraction of heated bovine plasma was used as the substrate. In preliminary experiments, the fraction was dissolved in 0.02M phosphate buffer (pH 7.5, 50 mg/ml) and 15 mg of it was assayed after the following treatments; 1) without incubation, 2) incubated by itself and 3) incubated with 100 ng of synthetic bradykinin. No contractile response was observed by the first two samples and it was confirmed that the fraction contained neither kinin nor plasma kallikrein. The third treatment was for detection of kininases, and even the sample incubated for 20 min gave the same intensity of response as that of the control which was bradykinin incubated without the fraction. The maximum kinin yield from this kininogen was determined in the response of 100 μg of trypsin, and the result is shown in Fig. 3. It was estimated that 1 mg of this kininogen yielded about 100 ng of bradykinin by the presence of an excess amount of trypsin.

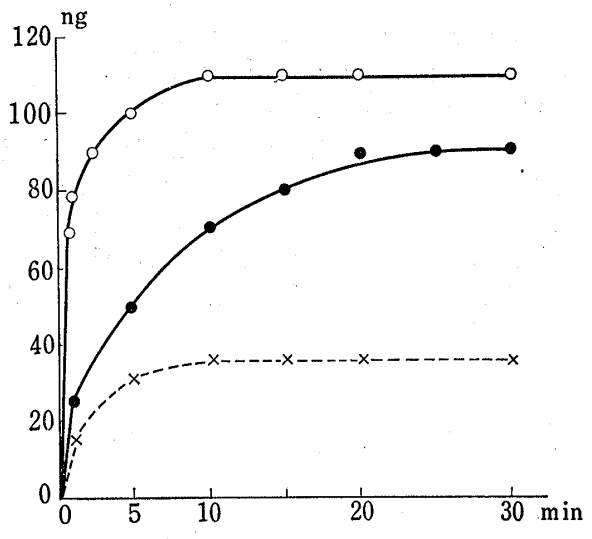

Fig. 3. Kinin Liberation from Bovine Kininogen

ordinate: Bradykinin equivalent yielded from 1 mg of the substrate.
abscissa: Incubation periods at 30°.
—○—: 1 mg of kininogen and 100 μg of trypsin
—●—: 5 mg of kininogen and 100 μg of trypsin
---×---: 5 mg of kininogen and 2 KU of most purified hog pancreatic kallikrein (KK-2)

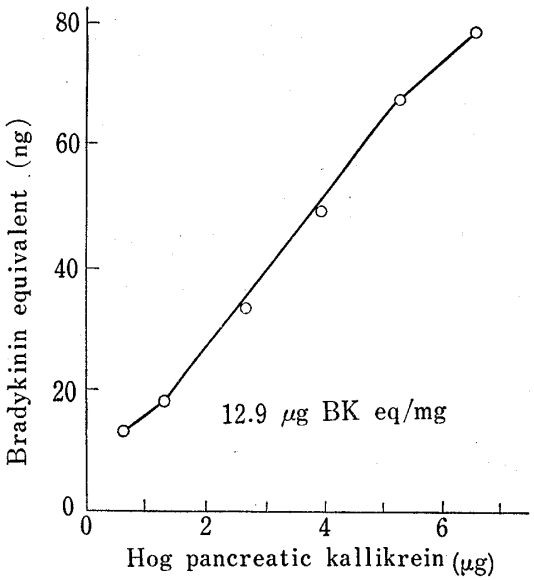

Fig. 4. Assay of the Kinin Forming Activity of Hog Pancreatic Kallikrein (KY-3)

Sample was incubated with 15 mg of bovine kininogen at 30° for 1 min, and kinin yielded was determined on an isolated guinea pig ileum.

Figure 4 shows a typical assay result determined by this method for hog pancreatic kallikrein (KY-3). From this result, the kinin forming activity of this sample was determined as 12.9 μg bradykinin equivalent per mg.

16) E.G. Erdös, "Advances in Pharmacology," Vol. 4, ed. by S. Garattini and P.A. Shore, Academic Press, New York and London, 1966, p.1.

We also compared the contractive activities of two preparations of synthetic bradykinin from different sources. Those activities of the preparations from Sandoz and Protein Research Foundation (Mino, Japan) were found to be nearly equal, and the relative potency of the former was calculated as 1.12±0.20.

Esterolytic Activity

Figures 5, 6, and 7 show the profiles of the determination of esterolytic activity of kallikrein (KY-3) with different assay methods. Its activity was determined as 1.80 μmoles TAME/min/mg when determined from the absorbance of the residual TAME as hydroxamate-ferric complex (Fig. 5), and 1.82 μmoles TAME/min/mg from that of the methanol liberated from the substrate by the sample (Fig. 6). Both results were in good agreement and similar results were also obtained in the activities of other samples (Table I).

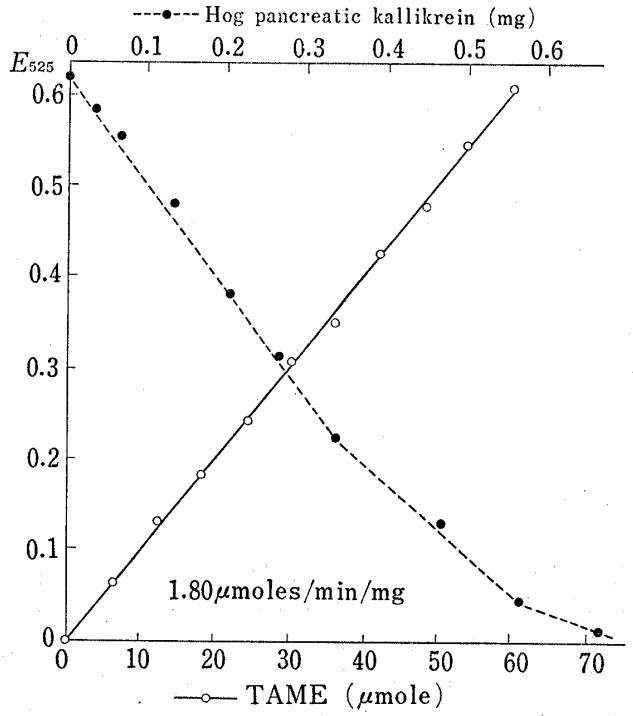

Fig. 5. Esterolytic Assay of Kallikrein (KY-3) by Measuring Residual TAME as Hydroxamate-Ferric Complex (30°, 60 min, pH 8.5)

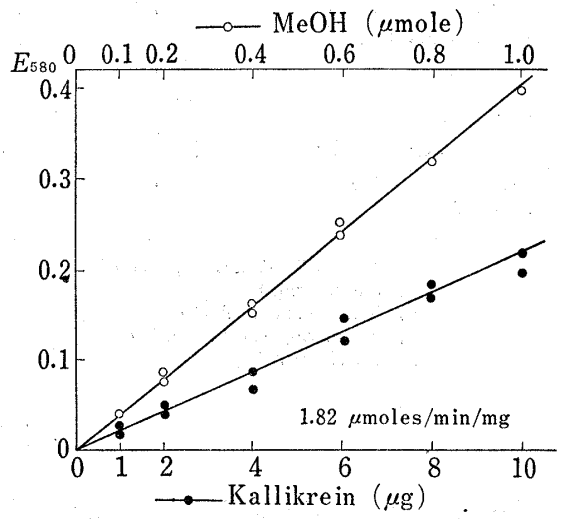

Fig. 6. Esterolytic Assay of Hog Pancreatic Kallikrein (KY-3) by Measuring Methanol Liberated from TAME as Formaldehyde-Chromotropic Acid Complex (30°, 30 min, pH 8.5)

At 37° this hog pancreatic kallikrein was found to have 38 EU/mg which corresponded to 2.38 μmoles TAME/min/mg when measured by the hydroxamate method and 2.10 when by the chromotropic acid method. The esterolytic activity on BAEE of the same sample was 13.9 μmoles/min/mg at 30° (Table I) and 10.9 at 25° (Fig. 7). Thus, the effect of temperature on assay results was significant as expected. Caseinolysis of various kallikrein preparations depended on their source and purity (Table I).

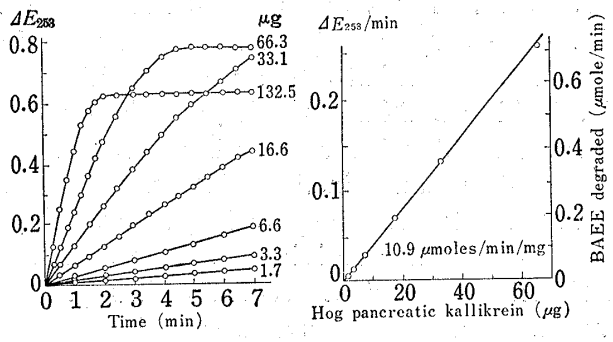

Fig. 7. Determination of Esterolytic Activity (BAEE) of Hog Pancreatic Kallikrein (KY-3) (pH 8.0, 25°)

In Table I, the activities of each sample have been summarized. The esterolytic activities are those determined at 30°, except the determination of EU.

TABLE I. Various Activities of Kallikreins Derived from Different Sources[a]

Kallikrein	Vasodilator (KU/mg)	Kinin forming (μg BK eq/mg)	Esterolytic				
			TAME			BAEE	caseinolytic (Kunitz U)
			EU[b]	H[c] (μmoles/min/mg)	C[d] (μmoles/min/mg)		
Hog pancreatic K							
KK-2[e]	1186	70.0	288	13.70	12.80	149.0	<0.01
KZ-6	146	25.0	45.5	1.46	1.23	14.7	0.60
KY-3	109	12.9	38.0	1.80	1.82	13.9	0.98
KU-9	100	10.0	49.5	1.00	1.01	10.6	0.34
701-F	23.25	1.2	25.0	0.32	0.34	2.6	0.31
Bayer kallikrein	4.9	0.26	4.8	0.20	0.19	0.92	0.63
KZC-150	998	160		12.20	13.10	171.5	<0.01
Human salivary K[e]	365	36.1	293	18.3	21.0	6.0	0.15
Human plasma K	0.008	0.003	0.38		0.01		
Human urinary K	1.9	0.04	1.4	0.06	0.08	0.03	
	21.9	2.80			1.04	0.36	
	200	27.0			9.90	2.80	
Guinea pig coagulating gl K[e]	333	4.4		9.7	8.30	2.40	<0.01
Trypsin (Sigma)	0.45	1.0	5000	135	130	31.2	3.13
Bradykinin	1800	1000					

a) some of the data were presented previously[13,17]
b) esterase unit[8]
c) hydroxamate method[3]
d) chromotropic acid method[18]
e) activity per E_{280}

Discussion

In the present investigation, the activities of kallikreins derived from various sources were determined. As shown in Table I, an increase of one activity generally accompanies elevation of other activities, however, it seems impossible to find definite conversion factors between the three main assays, *i.e.*, vasodilator, kinin forming and esterolytic determinations. Moreover, differences of the assay procedures, such as the incubation temperature and period in the esterolytic assay or the quality of kininogen in the kinin forming assay, caused significant variation in the results. Since these three activities seem to be essential for the determination of the kallikreins, it will be desirable to determine all of these activities by unified assay procedures. The authors would like to discuss some details of these assay procedures and also some enzymatic properties of kallikrein from this view point.

In the vasodilator assay, the quality of the standard kallikrein is an important factor. The KU is a well known unitage for kallikrein and this has been defined as the potency which causes a hypotensive response in dog that is equal to that given by a 5 ml aliquot of a dialysate of pooled human urine.[12] However, this standarization is not practical for routine assay, and as the standard preparation, we are using a hog pancreatic kallikrein preparation (701-F) whose vasodilator activity has been determined to be 23.25 KU/mg when compared to an authentic preparation. As shown in Fig. 1 and Table I, 1.0 μg of synthetic bradykinin gave 1.8 KU equivalent vasodilator activity, and the dose-response curve for bradykinin was parallel to that for the kallikrein. Therefore, it seems to be possible to use synthetic bradykinin as the standard and so to express the vasodilator activity in terms of equivalent bradykinin amount.

17) H. Moriya, N. Todoki, C. Moriwaki, and Y. Hojima, *J. Biochem.* (Tokyo), **69**, 815 (1971).

Arterial blood flow increase and hypotensive response by kallikreins or kinins are based on the same mechanism, but the measurement of blood flow increase was much more sensitive than the latter (Fig. 1). Consequently, since the former assay could be carried out with a smaller quantity of samples and shorter injection intervals also could be adopted, the arterial blood flow measurement seemed to be one of the most useful assays for kallikreins. Although these methods are excellent ones for kallikrein assay, a fall of the sensitivity during the assay procedure occurred often in dog. Consumption of the kininogen of the experimental animal might be one of the causes for this phenomenon, but a decrease of the response to synthetic bradykinin was also observed after repeated injections. Taking into account the change of the sensitivity, it is necessary to compare each response of a sample with that of a standard preparation.

Dogs were employed in our experiments. Other animals have been also used by other investigators and there are several convenient devices in the assay systems.[16,18] Though bradykinin and kallidin were found to give equal activity in vasodilator assay in dogs (Fig. 2), it has been reported that these kinins gave different intensity of vasodilatation in some other species.[16] Therefore, which kinin is yielded by the sample kallikrein also has to be considered on the assay in such animals.

Kinin forming activity or smooth muscle contractive activities can be expressed in terms of μg synthetic bradykinin equivalent as in this paper, or in μmole.[4b] The molecular weight of bradykinin is 1060, therefore it is easy to convert μg to μmole. In this assay system the substrate is an important component and should have neither active kallikrein, free kinin nor kininase as contaminants. The bovine pseudoglobulin fraction from heated plasma used as substrate kininogen in this investigation was found to be free from these contaminants. Acidification of plasma has been performed on the preparation of kininogen by Horton[19] and others, but Horton described neither the maximum release of kinin from the kininogen nor detail comparison of the properties of the kininogen and others prepared by different methods. We observed a lower kinin yield in acidified bovine plasma (pH 4.0 with 2% acetic acid) than that of the heated.[20] On the other hand, Jacobsen[21] reported the existence of two kinds of kininogen in plasma (HMW- and LMW-kininogen), and Suzuki and his collaborators[22] purified them separately from bovine plasma. According to ref. 21, plasma kallikrein yields bradykinin from HMW-kininogen, whereas glandular kallikreins, such as pancreatic, salivary or urinary kallikreins, act on the both kininogens and liberate kallidin from them. This might be also taken into account in the assay of kinin forming activity.

It was proposed[4b] that heated plasma derived from the same species as the kallikrein should be used. The use of its own kininogen will be meaningful in physiological or pathological investigations of kallikreins, but it is too crude and unsuitable for the accurate assay because of the occurrence of kininogen degradation during storage.[21b] Furthermore, rat and guinea pig have potent kininases in plasma which were still active even after heat treatment at 60° for 1 hr.[20] The bovine pseudoglobulin fraction used in this investigation seems to be better substrate for the routine assay of this activity.

An excess amount of kininogen should be used in this kinin forming assay. The maximum kinin yields from 1 mg of the above pseudoglobulin fraction were 100 and 35 ng of bradykinin equivalent by excess trypsin and by a purified hog pancreatic kallikrein (KK-2), respectively

18) K. Abe, T. Mouri, T. Seki, M. Suzuki, T. Takano, and K. Yoshinaga, *Experientia*, **24**, 455 (1968).
19) E.W. Horton, *J. Physiol.* (London), **148**, 267 (1959).
20) C. Moriwaki, in preparation.
21) *a*) S. Jacobsen, *Nature*, **210**, 98 (1966); *b*) *Idem*, *Brit. J. Pharmacol.*, **26**, 403 (1966); *c*) *Idem*, *ibid.*, **28**, 64 (1966); *d*) S. Jacobsen and M. Kuriz, *ibid.*, **29**, 25 (1967).
22) T. Suzuki, Y. Mizushima, T. Sato, and S. Iwanaga, *J. Biochem.* (Tokyo), **57**, 14 (1965); M. Yano, H. Kato, S. Nagasawa, and T. Suzuki, *ibid.*, **62**, 386 (1967); M. Yano, S. Nagasawa, K. Horiuchi, and T. Suzuki, *ibid.*, **62**, 504 (1967).

(Fig. 3). Pancreatic kallikrein is said to yield kallidin from the kininogen,[23] while trypsin liberates bradykinin,[24] and kallidin gave approximately 1/2—1/3 of the contractive response of bradykinin on guinea pig ileum (Fig. 2). The present results seem to be in good agreement with these facts. This kininogen can be defined as 0.1 mU/mg according to Webster's recommendation,[4b] in which 1 U of kininogen is defined as that amount of substrate which will yield 1 μmole of bradykinin equivalent in the presence of excess enzyme. Fifteen milligrams of this kininogen from which 1.5 μg of bradykinin (1.49 nmoles) and 0.525 μg of bradykinin equivalent (0.52 nmoles) could be yielded by excess trypsin and pancreatic kallikrein (KK-2), respectively, was used in this study, and this amount was sufficient for the sensitivity of the ileum preparation (Fig. 2).

One minute incubation of a kallikrein and the kininogen at 30° was performed in our assay. The maximum kinin release could not be obtained during this period (Fig. 3), and a satisfactory dose-response relation for the sample amount was found in this assay system (Fig. 4), so that 1 min incubation seems to be practical for the routine assay. Webster also proposed[4b] an arbitrary unit (mKaU) for kallikrein which was that amount of the enzyme which releases 1 nmole of bradykinin equivalent in 1 min at 30° from an amount of heated citrated plasma which contained 5 mU of kininogen. The hog pancreatic kallikrein (KY-3) yielded 12.9 μg bradykinin equivalent/mg in 1 min (Fig. 4) and it can be estimated as 12.8 mKaU/mg in accordance with the recommendation.

Though a good correlation was obtained between esterolytic and vasodilator activities of hog pancreatic kallikreins with varying purity, a great variation was found in their kinin forming activity. Kinin forming activity of 1 KU of them varied from 0.05 to 0.17 μg bradykinin equivalent (Table I). Some crude pancreatic kallikrein preparations contained potent kininase(s) as contaminant and the kinin formed by these samples became apparently lowered and disappeared after longer incubation. As concerns kininase, there are no standard assay method.[4b,25] In the present investigation, synthetic bradykinin and kininase sample were incubated in phosphate buffer pH 7.5 at 30° for up to 10 min, and then the remained bradykinin was assayed on guinea pig ileum. A pancreatic kallikrein preparation (701-F) decomposed 6 μg of bradykinin per mg in 1 min, and even a purified preparation (KY-3) still possessed a little kininase activity, whilst most purified ones (KK-2 and KZC-150) did not have kininase activity. It may be plausible that this kininase content caused the great variation of kinin forming activity, so that attention should be paid to kininase contamination in the assay sample.

It has been reported that 1,10-phenanthroline or EDTA-Ca were effective in inhibiting some kininases.[26] These chemicals were also used in the assay described above, and we noted the effect with the former agent on pancreatic kininases, but slight decrease of kinin forming activity was caused by the chemical on trypsin which was free from kininase. Details of this result will be discussed in another paper.

Esterolytic activity of kallikrein on various synthetic substrates was investigated by Webster and Pierce[3] and they found that the arginine esters were good substrates for kallikreins. TAME and BAEE have been favourably used for this purpose. An arbitrary unit (EU) was proposed by them, but the standard urinary kallikrein is required in the determination of this unit. Expression in terms of μmoles of the substrate degraded in 1 min is better to indicate this activity. As shown in Table I, the esterolytic activities of various kallikreins increased with the other activities, and good correlation was obtained between this activity and the vasodilator activity. However the conversion factor between the vasodilator and

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especially BAEE esterolytic activities varied according to the source of kallikrein, such as about 9 in hog pancreatic kallikrein and 60 in human urinary and salivary kallikreins. Recently Kutzbach and Schmidt-Kastner[7] reported that the conversion factor of FIP-unit (determined from BAEE hydrolyzing activity) to KU as obtained in biological assays was 6.37 for hog pancreatic kallikrein. Comparing the abilities on TAME and BAEE, the hog pancreatic kallikrein gave an activity ratio about 1: 10, whilst the ratio of other kallikreins deviated from this ratio. In human urinary and crude hog pancreatic kallikreins, EU almost coincided with their KU, but KU of the purified hog pancreatic kallikrein preparations were larger than EU. This seemed to be caused by elimination of trypsin-like enzyme contamination through purification processes, since trypsin is a potent TAME esterolytic enzyme. These results obtained in the esterolytic assays would be agreeable to those of other investigators.[10,27] Thus trypsin has potent esterolytic activity but poor vasodilator activity, and this would show the superiority of the vasodilator response in the kallikrein assay. Between vasodilator and kinin forming activities, as described above there was no satisfactory conversion factor through kallikreins from various sources.

The esterolytic activity on TAME determined by two different methods, *i.e.* measurement of the residual TAME as hydroxamate-ferric complex[3] and measurement of formed methanol with chromotropic acid,[13] were identical (Table I). Recently we developed another method for the esterolytic activity determination with 3-methyl-2-benzothiazolone hydrazone,[17] and this method also gave the same results. Therefore, TAME degrading activity of kallikrein under the same reaction condition might be comparable in despite of difference of methods.

It has been reported that kallikrein hydrolyzed traces of casein,[28] and our result is in good agreement with this fact (KK-2 and KZC-150, Table I). The caseinolytic activity of a preparation might be an indicator for the contamination of proteases, such as trypsin and chymotrypsin.

In the recommendation compiled by Webster[4] it has been suggested that kinin forming activity should be employed for standarization of kallikrein unit. The authors agree with her suggestion if kininase activity in sample can be sufficiently suppressed. There are a precise standard and a distinct unit for the kinin forming activity. It will be possible to compare the activity of various kallikreins from the same standpoint if adequate kininogen is used, and we would like to suggest that the bovine pseudoglobulin fraction from heated plasma will be one of such kininogens. However, the authors would like to propose the use of combined assays for kallikrein activity determination because of the lack of the information on relationship among these responses.

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