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Measurement of the Sugar Contents and Their Effect on the Electrophoretical Behaviors of Multiple Forms of Hog Pancreatic Kallikrein[1)]

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Kallikreins A and B isolated from hog pancreas showed vasodilator activities of 1350 and 1450 KU/mg (weight) respectively, and same esterolytic activity of 111 units/mg toward BzArgOEt. The sugar content of kallikrein B was roughly twice as much as that of kallikrein A in neutral hexose and glucosamine. Micro-heterogeneous forms B-2 and B-3 which are comprised in kallikrein B showed a 10 times difference in sialic acid content according to their acidities observed in isoelectric focusing. From these and other results obtained by gel plate isoelectric focusing, including the treatment with neuraminidase, it is suggested that the relative acidities on the electrophoresis of various forms of hog pancreatic kallikrein are depending upon the relative amounts of sialic acid and glucosamine in their molecules.

Keywords——hog pancreatic kallikrein; multiple forms of kallikrein; micro-heterogeneous forms; sugar contents; neuraminidase treatment; gel plate isoelectric focusing; isolation of kallikrein

Multiple forms of the kallikreins from hog pancreas, human urine, rat urine, rat submandibular gland, *etc.*, have been found and recently reviewed by Nustad *et al.*[3)] Most of these multiplicities have been observed by means of the technique of Ampholine isoelectric focusing. Among the above mentioned kallikreins, only hog pancreatic kallikrein[4)] and human urinary kallikrein[5)] have been separated into multiple forms (prepared) by means of electrophoresis, and diethylaminoethyl (DEAE) ion-exchangers and hydroxyapatite chromatographies.

As for the multiple forms of hog pancreatic kallikrein, in 1959 Moriya and Shimazawa observed two forms (called a_1 and a_2) on paper electrophoresis (see Fig. 3 in ref.[4a)]) of preparations obtained by electrophoresis with starch gel.[4a)] Afterwards, Moriya continued to study the kallikrein by using preparations obtained from the fraction containing only the form a_2 (form a_2 is guessed to be identical with form B from its behavior in electrophoretical migration).[6)] Thus, at that time our research on hog pancreatic kallikrein was directed toward

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1) Enzymes: Kallikrein (EC 3.4.21.8); Neuraminidase (EC 3.2.1.18). Abbreviations: N^{α}-Benzoyl-L-arginine ethyl ester, BzArgOEt; Kallikrein unit, KU.

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3) K. Nustad, K.M. Gautvik, and J.V. Pierce, "Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease," ed. by J.J. Pisano and K.F. Austen, U.S. Goverment Printing Office, Washington, D.C., 1977, pp. 77—92.

4) *a*) H. Moriya and E. Shimazawa, *Yakugaku Zasshi*, **79**, 374 (1959); *b*) E. Habermann, *Hoppe Seyler's Z. Physiol. Chem.*, **323**, 15 (1962); *c*) F. Fiedler and E. Werle, *Hoppe Seyler's Z. Physiol. Chem.*, **348**, 1087 (1967); *d*) T. Takami, *Seikagaku*, **41**, 777 (1969); *e*) C. Kutzbach and G. Schmidt-Kastner, *Hoppe Seyler's Z. Physiol. Chem.*, **353**, 1099 (1972); *f*) M. Zuber and E. Sache, *Biochemistry*, **13**, 3098 (1974); *g*) F. Fiedler, C. Hirschauer, and E. Werle, *Hoppe Seyler's Z. Physiol. Chem.*, **356**, 1879 (1975).

5) *a*) J.V. Pierce and K. Nustad, *Fed. Proc.* 31, 623 (Abs.) (1972); *b*) Y. Matsuda, K. Miyazaki, H. Moriya, Y. Fujimoto, Y. Hojima, and C. Moriwaki, *J. Biochem.*, **80**, 671 (1976).

6) *a*) H. Moriya and E. Shimazawa, *Yakugaku Zasshi*, **79**, 688 (1959); *b*) H. Moriya, *Yakugaku Zasshi*, **79**, 1216; 1390; 1451 (1959).

getting its highly purified preparation (form a_2)⁷⁾ without paying special attention to heterogeneity in the initial kallikrein, since the form a_1 was not only less in the content but also rather unstable compared with the form a_2.⁴ᵃ⁾ Recently because of the effective development of Ampholine isoelectric focusing, our interest was directed to multiple forms of hog pancreatic kallikrein shown by this technique.⁸⁾ And we have made sure and obtained the agreeable results to that hog pancreatic kallikrein is divided into forms A and B and both forms are composed of similar micro-heterogeneous forms with slightly different isoelectric points, as reported by other groups.⁴ᵉ,ᶠ⁾ Taking notice of the relationship between multiple forms and their sugar contents, in this work we separated highly purified forms A and B from the raw pancreas and analyzed the sugar compositions of them referring to their electrophoretical migration. Moreover, the relationship between micro-heterogeneous forms comprised in form B (which were prepared by isoelectric focusing) and their sugar compositions also referring to their electrophoretical behaviors, has been examined.

Experimental

Material

Hog pancreas was minced in a slaughter house, and the frozen pancreas was kindly supplied from Teikoku Hormone Mfg. Co. and stocked for several months at −20°. DEAE-cellulose (0.94 mEq/g), hydroxyapatite, Bio-Gel P-30 and P-100 were obtained from Tohoku Pulp Co., Seikagaku Kogyo Co. and Bio-Rad Labs., respectively; Sephadex G-75, DEAE-Sephadex A-50 (3.5 mEq/g) and QAE-Sephadex A-50 (3.0 mEq/g) from Pharmacia; N^{α}-benzoyl-L-arginine ethyl ester (BzArgOEt), Ampholine carrier ampholytes (pH 3.5—5), Cellogel membrane, neuraminidase (*Clostridium perfringens*, 0.6 unit/mg against muchin) from the Protein Research Foundation, Osaka, LKB-Produkter AB, Chemetron, Boehringer Mannheim GmbH, respectively. Other chemicals were of guaranteed reagent.

Methods

Assays of Kallikrein——The vasodilator activity of kallikrein was determined by measuring the increase of blood flow in the dog femoral artery, and compared with that produced by standard kallikrein (hog pancreatic, 23.25 KU/mg).⁹⁾ This activity is expressed in kallikrein unit (KU). Esterolytic activity of kallikrein toward BzArgOEt was determined at 25° and pH 8.0 in 0.05 M Tris-HCl by a spectrophotometric method at 254 nm, using a Hitachi 124 spectrophotometer.¹⁰⁾ One unit of esterolysis is defined as that amount of enzyme to hydrolyze 1 μmol of substrate per min (in the absence of calcium ions; final substrate concentration, approximately 1 mM).

Disc Electrophoresis——Disc electrophoresis was carried out in 10% (w/v) polyacrylamide gels (0.5 × 6 cm) at pH 8.9¹¹⁾ for the separation of kallikrein A and B. Electrophoresis was carried out at 2 mA per gel for 1.5 hr and protein was stained with 0.1% (w/v) Coomassie brilliant blue R-250 dissolved in 7% (w/v) trichloroacetic acid.

Preparative Isoelectric Focusing——A linear 0—50% (w/v) sucrose gradient density containing 1% (w/v) Ampholine (pH 3.5—5) at final concentration was prepared in an LKB Ampholine column (440 ml),¹²⁾ and electrophoresed for 40 hr at 500 V (constant) and 4°. The pH's of fractions collected from the column were measured at 4° with a Hitachi-Horiba F-5 pH meter with a combined electrode.

Gel Plate Isoelectric Focusing——This technique was performed following an instruction manual of LKB Produkter AB¹³⁾ or as described previously.¹⁴⁾ Six percent acrylamide solution containing 0.17% (w/v) N,N′-methylene-bis-acrylamide, 2% (w/v) Ampholine (pH 3.5—5), 12.5% (w/v) sucrose and small amounts of riboflavin and ammonium persulfate was polymerized in an LKB polymerization apparatus (0.2 × 11 × 24.5 cm), and the gel plate was fixed on an LKB-2117 Multiphor electrophoresis apparatus. Samples

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7) *a*) H. Moriya, J.V. Pierce, and M.E. Webster, *Ann. N.Y. Acad. Sci.*, **104**, 172 (1963); *b*) H. Moriya, A. Kato, and H. Fukushima, *Biochem. Pharmacol.*, **18**, 549 (1969).

8) *a*) H. Moriya, *J. Japan. Med. Assoc.*, **63**, 932 (1970); *b*) H. Moriya, Y. Matsuda, Y. Fujimoto, Y. Hojima, and C. Moriwaki, "Kininogenases-Kallikrein," ed. by G.L. Haberland and J.W. Rohen, F.K. Schattauer Verlag, Stuttgart, New York, 1973, pp. 37—42; *c*) Y. Hojima, Y. Matsuda, C. Moriwaki, and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), **23**, 1120 (1975).

9) C. Moriwaki, Y. Hojima, and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), **22**, 975 (1974).

10) G.W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, **16**, 570 (1955).

11) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).

12) O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, **20**, 820 (1966).

13) C. Karlsson, H. Davies, J. Öhman, and U.B. Andersson, "Application Note," Vol. 75, LKB-Produkter AB, Bromma, 1973, pp. 1—13.

14) Y. Hojima, M. Yamashita, N. Ochi, C. Moriwaki, and H. Moriya, *J. Biochem.* (Tokyo), **81**, 599 (1977).

were applied to filter paper squares (0.8×1 cm) which had been placed on the gel plate. Electrophoresis was carried out for 3 hr at 400 V (constant) under cooling with running water. The gel plate was stained with Coomassie brilliant blue R-250.

Cellogel Membrane Electrophoresis——A Cellogel membrane was pre-run for 30 min at 0.9 mA (constant)/cm in 0.08 M veronal buffer, pH 8.6. Afterwards, 1 μl of sample (0.01 esterolytic unit) was applied on the membrane in 0.8 cm length and run for 90 min under the above conditions. Esterolytic activity of the sample electrophoresed was detected by immersing the membrane in a solution of BzArgOEt-formazan staining system for 15 min at 37° as previously reported.[15]

Analyses of Sugars——Contents of fucose, mannose and galactose in kallikrein sample were analyzed by the gas chromatographic method of Lehnhardt and Winzler.[16] Sample (1.5 mg) in 1 ml of 1 N H_2SO_4 was hydrolyzed for 8 hr at 100° in an evacuated, sealed tube. Then the hexoses were changed to the respective alditol acetate derivatives, and amount of each derivative was determined by a Shimadzu GC-6A gas chromatograph. Glucosamine content of sample was determined by a Hitachi KLA-5 amino acid analyzer, after hydrolysis of sample (0.6 mg) in 0.9 ml of 4 N HCl for 6 hr at 100°. Sialic acid was determined as the amount of N-acetyl-neuraminic acid by the thiobarbituric acid method of Warren,[17] after hydrolyzing sample (1 mg) in 1 ml of 0.1 N H_2SO_4 for 1 hr at 80°.

Treatment with Neuraminidase——Kallikrein sample (0.4 mg) was incubated for 20 hr at 37° with 0.1 mg of neuraminidase in 1 ml of 0.05 M sodium acetate, pH 5.5.

Results and Discussion

Isolation of Kallikreins A and B

Step 1. Minced hog pancreas (frozen), 10 kg, was divided into 2 portions, and the following procedure was performed twice. The hog pancreas, 5 kg, was allowed to autolyze in 13 l of deionized water for 20 hr at room temperature (about 24°). The autolyzate was adjusted to pH 4.5 with 5 N and 1 N HCl, and was stirred for 2 hr. Acetone was then added dropwise in the stirred autolyzate to 20% (v/v) concentration in an ice bath, and this mixture was filtered through Toyo filter paper (No. 50) in a cold room (4°). Into the filtrate, 30 l of deionized water was added, and its pH was adjusted to pH 7.0 with 5 N and 1 N NaOH. Afterwards, 250 g of DEAE-cellulose (OH^- type) was added to the diluted filtrate, and stirred for 2 hr at room temperature, adjusting pH to 7 with addition of HCl. The DEAE-cellulose was recovered and packed in a column (7×60 cm bed size). The column was washed first with 0.01 M ammonium formate, pH 6.0, and kallikrein adsorbed on the cellulose was eluted with 0.6 M ammonium formate, pH 6.0. Two eluates from separate experiments were combined (4000 ml). The protein recovery in the combined eluates was 3.6% against the initial water extracts, and the recovery of vasodilator activity was 90%.

Step 2. Into the above solution, acetone was added dropwise up to 67% (v/v) concentration in an ice bath. The precipitate formed was collected by centrifugation, dissolved in 190 ml of 0.05 M Tris-HCl (pH 8.0), and filtered through a Sephadex G-75 column (4.5×50 cm) equilibrated with 0.3 M ammonium acetate (pH 6.0) at 4°. Fractions with a $A_{280}/A_{260} \geqq 1.2$ were pooled (350 ml).

Step 3. The pooled solution was applied on a column of DEAE-Sephadex A-50 (5×86 cm) equilibrated with 0.3 M ammonium acetate, pH 6.0. Following the condition of Takami,[4d] elution was performed at 4° by a convex gradient system formed by adding 0.7 M ammonium acetate (pH 6.0) to 1 l of equilibration buffer in a constant volume mixing chamber. As shown in Fig. 1, the kallikrein was separated into 2 peaks, kallikreins B and A, which were eluted at 0.46 and 0.52 M concentrations respectively.

Steps 4—6. Further purifications of kallikreins A and B were performed separately as follows. Kallikrein A was isolated by the successive chromatographies with QAE-Sephadex A-50 (repeated) and Bio-Gel P-100 equilibrated with 0.01 M ammonium formate, pH 6.0, and

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[15] Y. Fujimoto, H. Moriya, K. Yamaguchi, and C. Moriwaki, *J. Biochem.* (Tokyo), 71, 751 (1972).
[16] W.F. Lehnhardt and R.J. Winzler, *J. Chromatog.*, 34, 471 (1968).
[17] L. Warren, *J. Biol. Chem.*, 234, 1971 (1959).

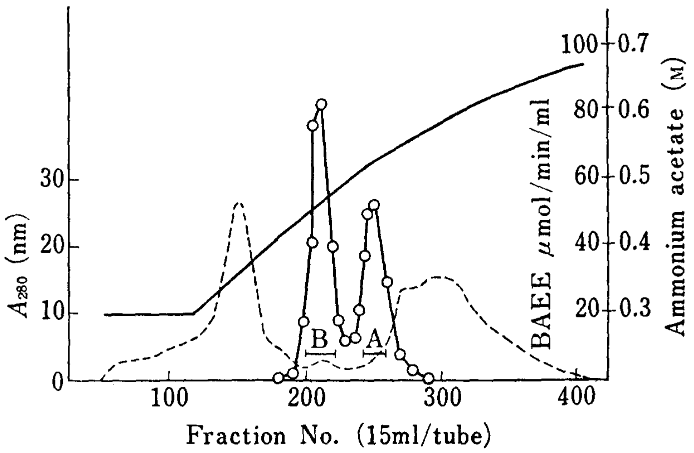

Fig. 1. DEAE-Sephadex A-50 Chromatography of Hog Pancreatic Kallikreins A and B

-----, Absorbance at 280 nm; —○—, BzArgOEt (BAEE) μmol/min/ml (esterolytic activity); ——, concentration of ammonium acetate (pH 6.0).

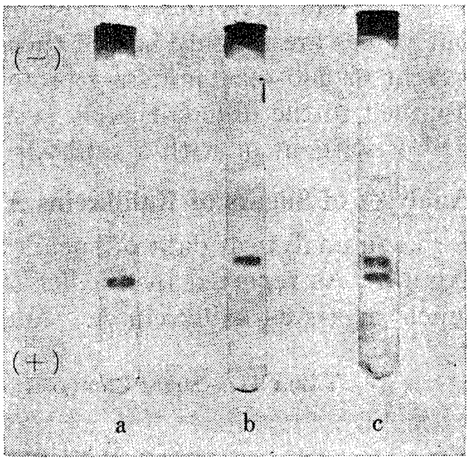

Fig. 2. Disc Electrophoresis of Kallikreins A and B

a, kallikrein A (18 μg), b; kallikrein B (16 μg); c, kallikrein A+B (A, 9 μg+B, 8 μg).

finally lyophilized. For kallikrein B, QAE-Sephadex A-50, hydroxyapatite and Bio-Gel P-100 were applied. The preparations of kallikreins A and B were homogeneous in disc electrophoresis with 10% polyacrylamide gels at pH 8.9 (Fig. 2), and showed different migration distances (A migrated faster) which were similar to those reported by Kutzbach and Schmidt-Kastner.[4e] As summarized in Table I, the recoveries of kallikreins A and B were 4—5% against step 1 in both vasodilator and esterolytic activities. Preparations of A and B showed 1350 KU/mg weight and 111 units/mg, and 1430 KU/mg and 111 unit/mg, respectively.

Table I. Isolation of Kallikreins A and B from Hog Pancreas

Step	Procedure	Total A_{280}	Vasodilator		Esterolytic	
			Total KU	KU/A_{280}	Total units	units/A_{280}
1	Extraction and DEAE-cellulose	300100	1001000	3.3	100000	0.33
2	Acetone (0—67%) and Sephadex G-75	35700	547000	15.3	42300	1.2
Kallikrein A						
3	DEAE-Sephadex A-50	506	—	—	11100	21.9
4	QAE-Sephadex A-50	114	—	—	7540	66.1
5	QAE-Sephadex A-50	66	—	—	4900	74.2
6	Bio-Gel P-100 and lyophilization	36 mg	48500	1350 mg	4000	111/mg
Kallikrein B						
3	DEAE-Sephadex A-50	630	—	—	16400	26.0
4	QAE-Sephadex A-50	130	—	—	10000	76.9
5	Hydroxyapatite	43	—	—	4300	100
6	Bio-Gel P-100 and lyophilization	38 mg	54300	1430 mg	4200	111/mg

The above vasodilator activities of kallikreins A and B are seemed to be almost equal to those reported by Kutzbach and Schmidt-Kastner (1050—1270 KU/mg weight)[18] and slightly larger than those of Zuber and Sache for kallikrein d_2 and d_1 (760 KU/mg weight, calculated from their data).[4f] However, in esterolysis, although our preparations showed the

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[18] C. Kutzbach and G. Schmidt-Kastner, "Kininogenases-Kallikrein," ed. by G.L. Haberland and J.W. Rohen, F.K. Schattauer Verlag, Stuttgart, New York, 1973, pp. 23—35.

equal specific activities to those of d_2 and d_1 (134 and 109 units/mg weight, respectively),[4f] our values are roughly half of those reported by two groups of West Germany (197 units/mg weight or 230—280 units/mg protein).[4e,g] This difference of esterolytic activities might be ascribed to the different assay systems, for example in substrate concentration (1 or 10 mM) and/or without or with a sulfhydryl compound in assay mixture.

Analyses of Sugars of Kallikreins A and B

The analytical data of sugars are presented in Table II, comparing with those of other groups. As reported by Fiedler *et al.*,[4g,19] our kallikrein B also contains roughly twice as much sugars as kallikrein A. And our values for the respective sugars in both kallikreins are almost equal to their values except for galactose, in which our values are double. On the other hand, for the sialic acid content, our values are in the range of 0—3 residues/mol which were agreed with those obtained for micro-heterogeneous forms of hog pancreatic kallikrein by Fritz *et al.*[20] However, our values are 15—20 times larger than those of Zuber and Zache.[4f]

TABLE II. Sugar Compositions of Kallikreins A and B (Residues/mol of Kallikrein)

Sugar	This work		Fiedler *et al.*[4g,19]		Zuber and Sache[4]	
	A	B	A	B	d_2	d_1
Fucose	0.41	1.3	0.5	1.1	Hexose	
Mannose	2.4	5.0	3.3	5.7	2.8	3.3
Galactose	1.8	3.3	0.8	1.5	Hexosamine	
Glucosamine	3.7	9.1	3.9	9.5	2.4	4.3
Sialic acid	0.25	0.78	—	—	0.016	0.04
Sugar contents	5.8%	12.6%	5.6%	11.5%	3.2%	4.6%

Our values have been calculated assuming that the molecular weights of our kallikreins A and B are 26800 and 28600 respectively, as reported by Fiedler *et al.*[4g,19]

The kallikrein forms d_2 and d_1 are probably identical with kallikreins A and B respectively, from their behaviors on DEAE-cellulose.[4f]

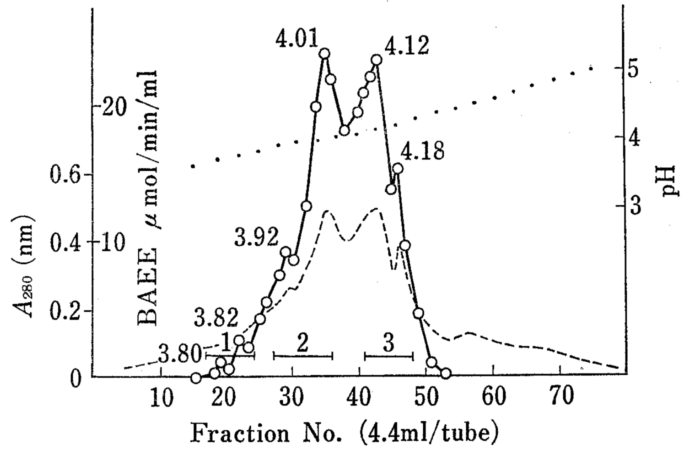

Fig. 3. Isoelectric Focusing of Hog Pancreatic Kallikrein B

Kallikrein B, 20 mg; focusing, 1% (w/v) Ampholine with pH 3.5—5, 40 hr, 500 V (constant), 4°.
------, absorbance at 280 nm; —○—, BzArgOEt (BAEE) μmol/min/ml (esterolytic activity); ·······, pH.

Analyses of Sugars of Micro-heterogeneous Forms comprised in Kallikrein B

To get micro-heterogeneous forms of kallikrein, kallikrein B (20 mg) was subjected to Ampholine isoelectric focusing. As shown in Fig. 3, the kallikrein was separated into 6 peaks in the range of pI 3.8—4.2 (kallikrein A also gave a similar focusing pattern in the same pI range). Every two peaks were combined, as indicated to be 1 (pI 3.8), 2 (pI 3.9—4) and 3 (pI 4.1—4.2) in Fig. 3. The combined solutions were separately filtered through Bio-Gel

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19) *a*) F. Fiedler, *Methods Enzymol.*, **45**, 289 (1976); *b*) F. Fiedler, "Chemistry and Biology of the Kallikrein–Kinin System in Health and Disease," ed. by J.J. Pisano and K.F. Austen, U.S. Goverment Printing Office, Washington, D.C., 1977, pp. 93—95.

20) H. Fritz, I. Eckert, and E. Werle, *Hoppe Seyler's Z. Physiol. Chem.*, **348**, 1120 (1967).

P-30 columns equilibrated with 0.01 M ammonium formate, pH 6.0 and lyophilized. These preparations were named forms B-1, B-2 and B-3, and weighed 1, 8 and 8 mg, respectively. Sugar analyses of the forms B-2 and B-3 are shown in Table III (The analysis for B-1 could not be done because of its small amount and also because it had been used in other limited experiments). The contents of fucose, mannose and glucosamine of forms B-2 and B-3 are roughly equal each other, and those values are nearing the respective values of kallikrein B. Only the galactose content of B-2 is about twice as much as that of B-3, while between the sialic acid content of B-2 and B-3, a 10-times difference is found. This matter can be ascribed to their relative acidities of molecules shown in Fig. 3. And therefore, it would be guessed that the form B-1 contains much amount of sialic acid.

TABLE III. Sugar Compositions of Forms B-2 and B-3
(Residues/mol of the Form)

Sugar	Kallikrein B	Form B-2	Form B-3
Fucose	1.3	1.7	1.2
Mannose	5.0	3.8	5.1
Galactose	3.3	1.2	0.52
Glucosamine	9.1	7.5	7.1
Sialic acid	0.78	0.58	0.046
Sugar contents	12.6%	9.5%	8.8%

The values for forms B-2 and B-3 have been calculated from an assumption that the molecular weights of them are 28600, which is the molecular weight of kallikrein B.[4g] The values for kallikrein B are cited from Table II.

Gel Plate Isoelectric Focusing and Cellogel Membrane Electrophoresis of Kallikreins A and B

In the gel plate isoelectric focusing, protein patterns of kallikreins A and B were almost same (Fig. 4). However, after treatment with neuraminidase, although more acidic microheterogeneous forms in both kallikreins were converted to less acidic ones, there was a difference observed between their resulted protein patterns–the treated kallikrein A showed a major protein band relatively more acidic than that from the treated kallikrein B (Fig. 4). This might be caused by the different amounts of glucosamine of these kallikreins.

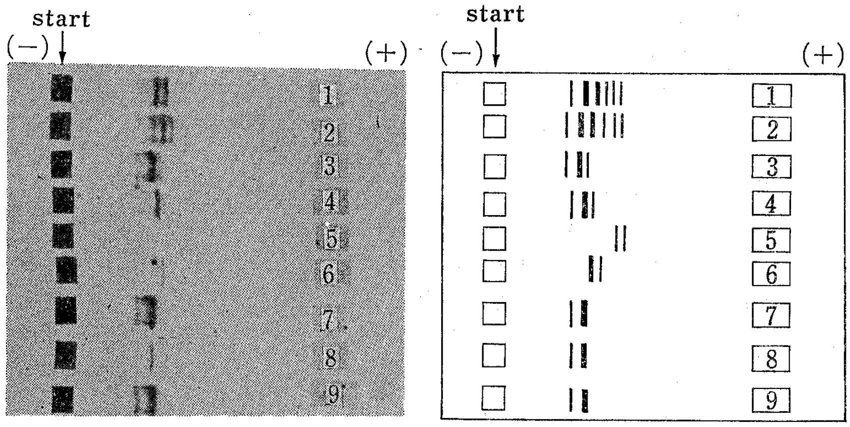

Fig. 4. Gel Plate Isoelectric Focusing of Kallikreins

1) kallikrein A; 2) kallikrein B; 3) kallikrein B treated with neuraminidase; 4) kallikrein A treated with neuraminidase; 5) kallikrein B-1; 6) kallikrein B-2; 7) kallikrein B-2 treated with neuraminidase; 8) kallikrein B-3; 9) kallikrein B-3 treated with neuraminidase.

The amounts of the kallikreins applied, 10—20 μg; Polyacrylamide gel 6% (w/v) (0.2×11×24.5 cm); Focusing, 3 hr at 400 V (constant); Staining, 0.1% (w/v) Coomassie brilliant blue R-250.

Kallikrein A contained about a half amount of glucosamine of that of kallikrein B. Similar focusing protein patterns have been observed in gel tubes by Kutzbach and Schmidt-Kastner (Fig. 8 of ref.18)).

The micro-heterogeneous forms from kallikrein B, B-1, B-2 and B-3 showed the protein patterns clearly separated one another in the focusing (Fig. 4). After treatment with neuraminidase, the form B-2 showed a protein pattern shifted to cathode side, being divided into a major and a minor bands, while in the case of the form B-3 the resulted protein pattern was not shifted and almost the same even after treatment with neuraminidase (Fig. 4). Therefore, in conclusion, these results suggest that the minor protein bands newly appeared after treatment of B-2 might be really free proteins from sialic acid although there were almost no shifted protein bands of the treated form B-3 because of its quite small content of sialic acid as indicated in Table III. Also on the Cellogel membrane electrophoresis combined with a BzArgOEt-formazan system, were observed some differences of the migration distances between intact and treated kallikrein and among those of forms B-1—B-3 (Fig. 5). This membrane electrophoresis might be a useful technique to detect such enzymes as kallikrein and other arginine esterases.

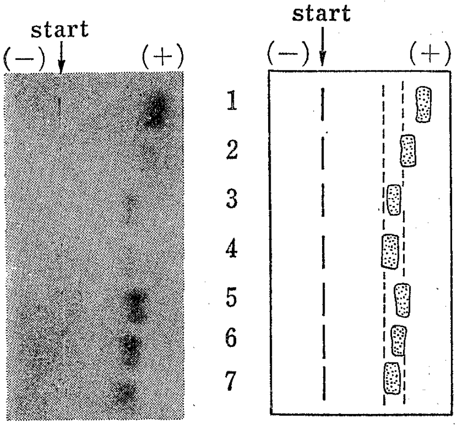

Fig. 5. Cellogel Membrane Electrophoresis of Kallikreins

1) kallikrein A; 2) kallikrein A treated with neuraminidase; 3) kallikrein B; 4) kallikrein B treated with neuraminidase; 5) kallikrein B-1; 6) kallikrein B-2; 7) kallikrein B-3.

The amounts of the kallikreins applied, 80—100 µg; Cellogel membrane (5×18 cm); Electrophoresis, 0.9 mA/cm, 90 min, at pH 8.6; Staining, immersed in a solution of BzArgOEt-formazan staining system15) for 15 min at 37°.

According to the studies of Fiedler *et al.*4g) kallikreins A and B have identical amino acid compositions except an excess of 3 amide ammonia residues in kallikrein B. However, for sugar content, our preparation of kallikrein B showed about twice as much neutral hexose and glucosamine as kallikrein A, as reported by them. This difference has been described by them to be sufficient for the separation of these kallikreins on anion-exchanger chromatography and electrophoresis. Fiedler *et al.* did not observe the sialic acid content of these kallikreins because they isolated these kallikreins after neuraminidase treatment. In our determination of the sialic acid, kallikrein B showed 3 times as much content as kallikrein A (Table II). As for the comparison of the sialic acid contents of micro-heterogeneous forms B-2 and B-3 which were divided from kallikrein B, there was much more difference (10 times), depending upon their pI's (Table III and Fig. 3). On the other hand, the glucosamine contents of these forms were almost equal. From these results and also including the isoelectric patterns shown by kallikreins A and B and those which had been treated with neuraminidase (Fig. 4), it would be suggested that the relative acidities of various forms of the kallikrein are depending upon the relative amount of sialic acid and glucosamine in their molecules. For our previous preparations of hog pancreatic kallikrein, we like to mention again as follows.8c) The preparation of ref.4a,6,7a) obtained by starch gel electrophoresis was the main fraction contained only form a_2 (more slowly than a_1 in migration), therefore this preparation might correspond to kallikrein B. The preparation of ref.7b) will be guessed to be the mixture of kallikreins A and B, because it was purified by hydroxyapatite, on which the separation of the kallikrein forms has not been successful as yet.19a) And the kallikrein preparation, which was purified by preparative disc electrophoresis with 7% polyacrylamide gel,21) must be the mixture of kallikreins A and B, because the starting material employed

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21) Y. Hojima, C. Moriwaki, and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), 23, 1128 (1975).

in this electrophoresis was a product by the acrinol method of Moriya,[22) from which Takami had isolated the two forms of kallikrein.[4d)

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22) H. Moriya, *Yakugaku Zasshi*, **79**, 32 (1959).