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Studies on Heterogeneous Components of Hog Pancreatic Kallikrein —"Possible Role of the Neuraminic Acid Residues"—

Masahiko Ikekita,[1a] Hiroshi Moriya,[1a] Seiichi Ozawa,[1b] and Kazuyuki Kizuki[1a]

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo,[1a] 12, Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan and Research Institute, Daiichi Seiyaku Co., Ltd.,[1b] 1-6-13, Kitakasai, Edogawa-ku, Tokyo 132, Japan

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Fucose, mannose, galactose, N-acetylglucosamine and neuraminic acid were detected as structural sugars of the purified hog pancreatic kallikreins A and B. Kallikrein B contained roughly twice as much sugar as kallikrein A (A, 4.56; B, 8.40% by weight). Kallikreins A and B were further divided into 3 (A-I, A-II and A-III) and 6 (B-I, B-II, B-III, B-IV, B-V and B-VI) micro-heterogeneous components, respectively. The sugar contents of B-III and B-IV (the main micro-heterogeneous forms of B) were also about twice as much as that of A-II (one of the micro-heterogeneous forms of A). As for the content of neutraminic acid, A (more acidic than B) contained less than B. On the other hand, the contents of neutraminic acid in the micro-heterogeneous components from A and B were different from each other, and the more acidic micro-heterogeneous forms were richer in neuraminic acid. After treatment with neuraminidase, the more acidic micro-heterogeneous forms shifted to the positions of the less acidic ones. These observations suggested that the neuraminic acid content in the molecule was the main factor permitting the separation of the micro-heterogeneous forms by isoelectric focusing. After the reduction of kallikreins A and B with 2-mercaptoethanol, two protein bands were detected in each case on polyacrylamide gel electrophoresis. However, although staining of the carbohydrate moiety of the reduced B with PAS reagent also showed two bands identical with the protein bands, only one of the protein bands of the reduced A was stained for carbohydrate. Thus, kallikreins A and B both consist of two peptide chains, but one of the peptide chains of A has no carbohydrate moiety, whereas carbohydrate moieties are bound to both peptide chains in the case of kallikrein B. The Km values and optimum pH's of kallikreins A and B, their micro-heterogeneous forms (A-II, A-III, B-IV and B-V) and asialo-kallikreins A and B for Bz-Arg-OEt hydrolysis were closely similar. Asialo kallikreins A and B were less stable than intact sialo-A and -B at 80° during incubation for 1 hr. Thus, neuraminic acid appears not to be related to the enzymatic activity, but dose influence the stability of the enzyme molecules.

Keywords——hog pancreatic kallikrein; multiple forms of kallikrein; micro-heterogeneous forms; carbohydrate contents of kallikreins; neuraminic acid; isoelectric focusing; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; asialo-kallikrein

Recently, it has been revealed that the kallikreins, such as human urinary, hog pancreatic, rat submandibular and some other glandular kallikreins, have multiple forms which are usually separable on Ampholine isoelectric focusing.[2] However, most of their multiple forms have not been separately prepared except for human urinary[3] and hog pancreatic[4] kallikreins. Multiple forms of hog pancreatic kallikrein were initially observed by Moriya and Shimazawa (labelled a_1 and a_2 on the paper electrophoretogram in Fig. 3 of ref. 4*a*), being separable by electrophoresis on starch gel.[4a] Afterwards, the presence of these two forms of kallikrein was confirmed and they were separated by other groups[5,6] and named kallikreins A and B. These two kallikreins were further separated into several microheterogeneous forms having different isoelectric points.[6,7] It was also found that hog pancreatic kallikrein contains carbohydrate components.[6,8-10] In our previous paper,[10] we investigated the carbohydrate contents of kallikreins A, B and some of the partially purified micro-heterogeneous forms of

kallikrein B, and discussed the relationship between the multiple forms of kallikrein and their carbohydrate contents.

We have recently obtained larger amounts of highly pure micro-heterogeneous preparations, so that more detailed studies become possible. The present paper deals with the carbohydrate contents and the compositions of the purified micro-heterogeneous forms from both kallikreins A (A-II and A-III) and B (B-III, B-IV and B-V), obtained by the isoelectric focusing. The carbohydrate-carrying peptide chains of kallikreins A and B were also examined by SDS-polyacrylamide gel electrophoresis in order to elucidate the relationship between the compositions and the function(s) of the neuraminic acid residues in kallikreins A and B, and their micro-heterogeneous forms.

Some preliminary results of this work have already been reported.[11]

Materials and Methods

Materials

Hog pancreas, which was kindly supplied by Teikoku Hormone Mfg. Co., Tokyo, was minced and stored at −20°. DEAE-cellulose (0.88 mEq/g) was obtained from Serva Feinbiochemica GmbH and Co., Heidelberg; Sephadex G-50 (medium) and DEAE-Sephadex A-50 (3.0±0.4 mEq/g) from Pharmacia Fine Chemicals Inc., Sweden; Bio-Gel P-30, P-100 and Amberlite AG-1X8 ($HCOO^-$ type, 200—400 mesh, 3.2 mEq/g) from Bio-Rad Labs., California; Dowex 50W-X8 ($HCOO^-$ type, 200—400 mesh, 4.8 mEq/g) from Dow Chemical Co., California; N^{α}-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt), and Ampholine carrier ampholites (pH 3.5—5.0) from the Protein Research Foundation, Osaka, and LKB Produkter AB, Sweden, respectively; neuraminidase (*Streptococcus sp. IID6646*, 50 U/mg against N-acetyl-neuraminyl (2→3) lactose) and endo-β-N-acetylglucosaminidase H (endoglycosidase H, *Streptococcus griseus*, 30 U/mg against [^{14}C]-acetyl-Asn-$GlcNAc_2$-Man_6) from Seikagaku Kogyo Co., Tokyo; neuraminic acid from Nakarai Chemicals, Ltd., Kyoto. Other chemicals were of guaranteed reagent grade.

Methods

Assays of Kallikrein——The vasodilator activity of kallikrein was determined by measuring the increase of blood flow in the dog femoral artery and comparing it with that produced by the standard kallikrein.[12] The activity was expressed in terms of the kallikrein unit (KU). Esterolytic activity of kallikrein towards Bz–Arg–OEt was determined at 25° in 0.1 M Tris-HCl (pH 8.0) by a spectrophotometric method at 254 nm, using a Hitachi 124 electrophotometer.[13] The final concentration of substrate was 0.5 mM. One unit was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per min (in the absence of calcium ions).

Protein Determination——The absorbance at 280 nm in a 1.0 cm wide cuvet was routinely measured to estimate protein concentrations of various solutions. Dry weight of proteins was estimated after lyophilization of salt-free aqueous solutions.

Conductivity Determination——The conductivity of the fractions from DEAE-Sephadex A-50 chromatography was measured at 25° with a model CM-20A conductivity meter and a type CG-501 PL cell, Towa Electronics Ltd., Tokyo.

Disc Electrophoresis——Disc electrophoresis was carried out in 10% (w/v) polyacrylamide gels (0.5×6.0 cm) for 1.5 hr at pH 8.9 (2 mA per gel).[14] Proteins were stained with 0.1% (w/v) Coomassie brilliant blue R-250 dissolved in 7% (w/v) trichloroacetic acid.

Preparative Isoelectric Focusing——Linear sucrose density gradients, 0—65% (w/v), containing 1% (w/v) Ampholine (pH 3.5—5.0) (final concentration) were prepared in LKB Ampholine columns (110 ml and 440 ml),[15] and electrophoresis was carried out for 40 hr at 3 W (constant) and 4°. The pH's of fractions collected from the column were measured at 4° with a Hitachi-Horiba F-5 pH meter, using a combined electrode, model 6028.

Gel Plate Isoelectric Focusing——Isoelectric focusing was performed according to an instruction manual of LKB Produkter[16] or as described previously.[17] Six percent acrylamide solution containing 0.17% (w/v) N,N′-methylene-bis-acrylamide, 2% (w/v) Ampholine (pH 3.5—5.0), 12.5% (w/v) sucrose, 0.04% (w/v) riboflavin and 0.05% (w/v) ammonium persulfate was polymerized (0.2×12.0×26.0 cm). Sample were applied to paper squares (LKB 2117-106, 0.1×0.6×0.5 cm) which had been placed on the gel plate. Electrophoresis was carried out for 3 hr at 25 W (constant) under cooling with running water. The gel plate was stained with 0.01% (w/v) Coomassie brilliant blue R-250 dissolved in 30% (v/v) ethanol containing 3% (w/v) sulfosalicylic acid and 10% (w/v) trichloroacetic acid.

SDS-Polyacrylamide Gel Electrophoresis——SDS-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn.[18] Some samples were treated with 0.04% (v/v) 2-mercaptoethanol in 0.01 M Tris-HCl buffer, pH 7.4 containing 1% (w/v) SDS for 30 min at 37°. Molecular-weight marker protein were obtained from Daiichi Pure Chemicals Co. Ltd., Tokyo.

Analysis of Sugars——Contents of fucose, mannose and galactose in kallikrein molecules were analyzed by the gas chromatographic method of Spiro.[19] A 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 converted to the corresponding alditol acetate derivatives, and the amount of each derivative was determined by means of a Shimadzu GC-6A gas chromatograph, Tokyo. The content of N-acetylglucosamine was determined on a Hitachi automatic liquid chromatograph, model 034, with a column of Hitachi custom ion exchange resin #2611, after hydrolysis of the sample (0.6—0.8 mg) with 1.0 ml of 4 N HCl for 8 hr at 100°. Neuraminic acid was determined as the amount of N-acetyl-neuraminic acid by the periodate-resorcinol method of Jourdian *et al.*[20]

Treatment with Neuraminidase——Kallikreins (0.2 mg) were incubated for 24 hr at 37° with 0.1 U of neuraminidase in 0.01 ml of 1.0 M sodium acetate, pH 6.5, containing 0.1 M calcium chloride and 0.5% (w/v) bovine serum albumin.

Treatment with Endoglycosidase H——Neuraminidase-treated kallikreins (0.1 mg) were incubated with 0.005 U of endoglycosidase H in 0.005 ml of 1.0 M sodium citrate, pH 5.0, for 24 hr at 37°.

Enzyme Purification——Step 1. Water Extraction and DEAE-cellulose Chromatography (Batch Method): Minced hog pancreas (frozen), 40 kg, was divided into 8 portions and the following procedures were performed on each portion separately. The hog pancreas (5 kg) was allowed to autolyzed in 15 l of deionized water for 20 hr at room temperature. The autolyzate was adjusted to pH 4.5 and stirred for 2 hr. Acetone was then added dropwise into the stirred autolyzate to make 20%(v/v) concentration in an ice bath, and this mixture was filtered through nylon net. The filtrate was centrifuged (7000 rpm, for 20 min at 4°) and the supernatant was further filtered through Toyo filter paper (No. 50). The resulting clear solution was adjusted to pH 7.0 and dialyzed against tap water for 16 hr at room temperature. Next, 90 l of deionized water was added to the dialyzed solution, and its pH was readjusted to 7.0. Afterwards, 100 g (dry weight) of DEAE-cellulose was added to the solution, and the mixture was stirred for 2 hr at room temperature. The DEAE-cellulose was recovered and packed in a column (4×60 cm bed size). The column was washed first with 0.02 M ammonium acetate, pH 6.0, until the absorbance at 280 nm fell below 0.01, then kallikrein was eluted with 0.6 M ammonium acetate, pH 6.0. Fractions containing kallikrein were pooled and dialyzed against 0.01 M Tris-HCl, pH 8.0, for 16 hr at 4°, then concentrated.

Step 2. First Acetone Fractionation: Acetone was added to the above solution and the precipitate at 40—75% (v/v) acetone was collected. The precipitate was dissolved in 0.01 M Tris-HCl, pH 8.0, to make a final concentration of 50 A_{280}/ml.

Step 3. Second Acetone Fractionation: Acetone fractionation of the above solution was carried out again. The precipitate at 50—80% (v/v) acetone was dissolved in 300 ml of 0.3 M ammonium acetate, pH 6.0.

Step 4. Sephadex G-50 Filtration: This solution was filtered through a Sephadex G-50 column (4.5×140 cm) equilibrated with 0.3M ammonium acetate, pH 6.0 at 4°. Fractions with $A_{280}/A_{260} \geqq 1.2$ were pooled and dialyzed against the same buffer (5.0 l×4).

Step 5. DEAE-Sephadex A-50 Chromatography: The dialysate was applied to a column of DEAE-Sephadex A-50 (2.5×90 cm) equilibrated with 0.3 M ammonium acetate, pH 6.0. Under the conditions of Takami,[4d] elution was performed at 4° with a linear gradient system formed by adding 0.7 M ammonium acetate, pH 6.0, to 1 l of the equilibration buffer in a constant-volume mixing chamber. As shown in Fig. 1, kallikrein was separated into 2 peaks, kallikreins B and A. which were eluted at conductivities of 34 and 39 mV/cm, respectively.

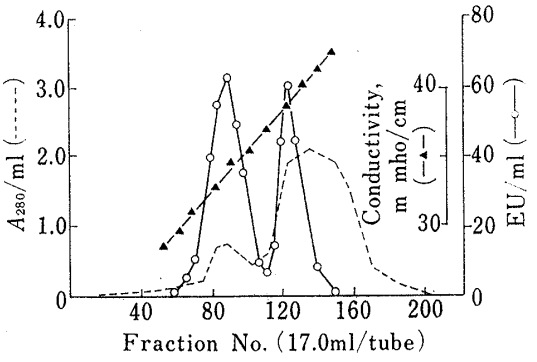


Fig. 1. Separation of Hog Pancreatic Kallikreins A and B by DEAE–Sephadex A-50 Chromatography

Steps 6 and 7. QAE-Sephadex A-50 and Bio-Gel P-100 Chromatographies: Further purifications of kallikreins A and B were performed separately, mainly according to the methods reported previously.[10] Kallikreins A and B were each isolated by successive chromatographies on QAE-Sephadex A-50 and Bio-Gel P-100 equilibrated with 0.3 M ammonium acetate, pH 6.0, and 0.01 M ammonium formate, pH 6.0, respectively, and finally lyophilized.

Results and Discussion

Purity of Hog Pancreatic Kallikreins A and B

As shown in Table I, the final recoveries of kallikreins A and B from step 1 (DEAE-cellulose eluate) were 20—25% as estimated in terms of esterolytic activity. This purification method was simple and the recovery was higher than that of the previous method.[10] Pre-

Table I. Purification of Hog Pancreatic Kallikrein

Step		Total EU	Total A_{280}	EU/A_{280}	EU Yield (%)
1. Minced Hog Pancreas (40 kg)		—	—	—	—
2. DEAE–Cellulose		178000	416000	0.428	100
3. 1st. Acetone Frac.		164000	181000	0.907	92.0
4. 2nd. Acetone Frac.		151000	145000	1.04	84.6
5. Sephadex G-50		140000	93800	1.50	78.7
6. DEAE–Sephadex A-50	(B)	66000	840	78.7	37.1
	(A)	48700	3600	13.6	27.3
7. QAE-Sephadex A-50	(B)	54200	624	87.0	30.4
	(A)	41500	558	74.4	23.3
Bio–Gel P-100	(B)	41800	400	105	23.4
	(A)	37900	371	102	21.3

Final preparations of A and B showed activities of 1350 KU/mg and 1400 KU/mg, respectively.

parations of A and B showed activities of 1350 KU/mg and 102 EU/A_{280}, and 1400 KU/mg and EU/A_{280}, respectively. The above vasodilator activities of kallikreins are almost equal to those reported in our previous paper[10] and those of another group.[21] The final preparations of kallikreins A and B were homogeneous both in disc electrophoresis with 10% (w/v) polyacrylamide gels at pH 8.9 (Fig. 2) and in SDS-polyacrylamide gel electrophoresis with 7.5% (w/v) polyacrylamide gels containing 1% (w/v) SDS at pH 7.4 (Fig. 3).

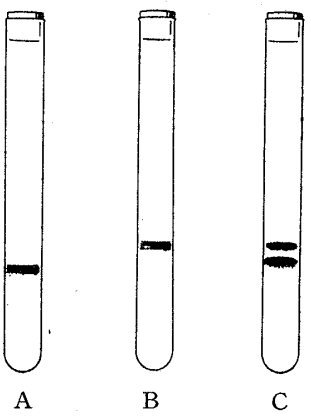


Fig. 2. Disc Electrophoresis of Kallikreins A and B

A, kallikrein A (10 μg); B, kallikrein B (10 μg); C, mixture of kallikreins A and B (10 μg+10 μg). After electrophoresis, gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250.

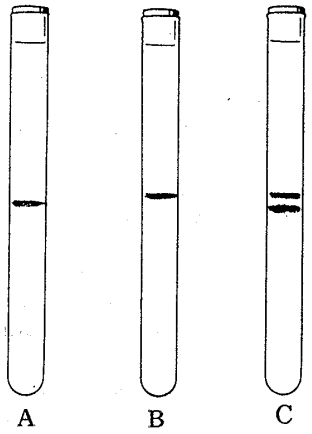


Fig. 3. Electrophoregrams of Kallikreins A and B on SDS–Polyacrylamide Gel

A, kallikrein A (10 μg); B, kallikrein B (10 μg); C, mixtures of kallikreins A and B (10 μg+10 μg). After electrophoresis, gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250.

Preparative Isoelectric Focusing of Kallikreins A and B

In order to further characterize the heterogeneous forms (kallikreins A and B of step 7), preparative isoelectric focusing was performed (Fig. 4). Three peaks having Bz-Arg-OEt esterolytic activity with pI's of 4.05, 4.18 and 4.28 were obtained from kallikrein A. The fractions shown in Fig. 4-A were separately pooled and were named A-I, A-II and A-III, as indicated (Fig. 4-A). The pooled preparations (A-I, A-II and A-III) had activities of 56.3, 170.0 and 156.0 EU/A_{280}, respectively. On the other hand, as shown in Fig. 4-B, kallikrein B was separated into 6 peaks with pI's of 4.00, 4.05, 4.10, 4.14, 4.30 and 4.50, which were named B-I, B-II, B-III, B-IV, B-V and B-VI, respectively. The main fractions, B-III, B-IV and B-V, showed activities of 109.0, 141.0 and 114.0 EU/A_{280}, respectively. The micro-

heterogeneous fractions obtained from both kallikreins A and B were separately dialyzed against 0.01 M ammonium formate, pH 6.0, for 20 hr at 4° and separately filtered through a Bio-Gel P-30 column equilibrated with the same buffer, then lyophilized.

The main fractions (A-II, A-III, B-III, B-IV and B-V) were employed in the following experiments for sugar analysis and analysis of enzymatic properties because other minor fractions, A-I, B-I, B-II and B-VI, were obtained in amounts too small to permit analysis.

Carbohydrate Compositions of Kallikrein A, B and their Micro-Heterogeneous Forms

The carbohydrate compositions of kallikreins A, B and their micro-heterogeneous forms (A-II, A-III, B-III, B-IV and B-V) are summarized in Table II. The neutral sugar, fucose (Fuc), mannose (Man) and galactose (Gal), content of kallikrein B was about twice that of kallikrein A. This observation is in accord with both that reported in our previous paper[10]

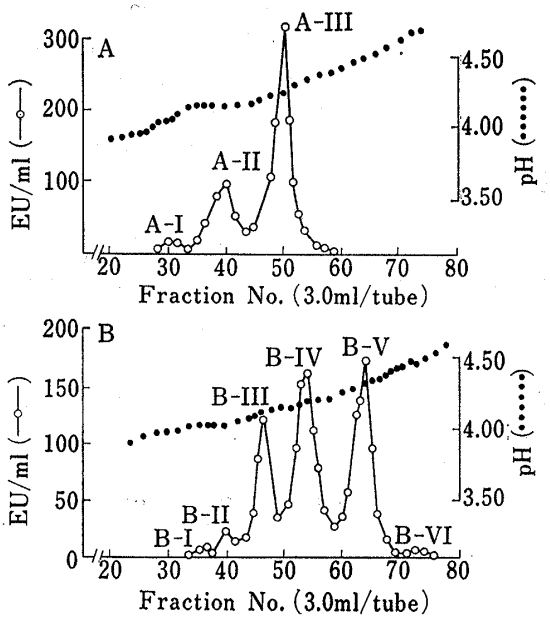


Fig. 4. Isoelectric Focusing of Hog Pancreatic Kallikreins A and B

A, kallikrein A (40 mg); B, kallikrein B (54 mg); Focusing was carried out in 1% (w/v) Ampholine at pH 3.5—5.0, for 40 hr at 3 W (constant), at 4°.

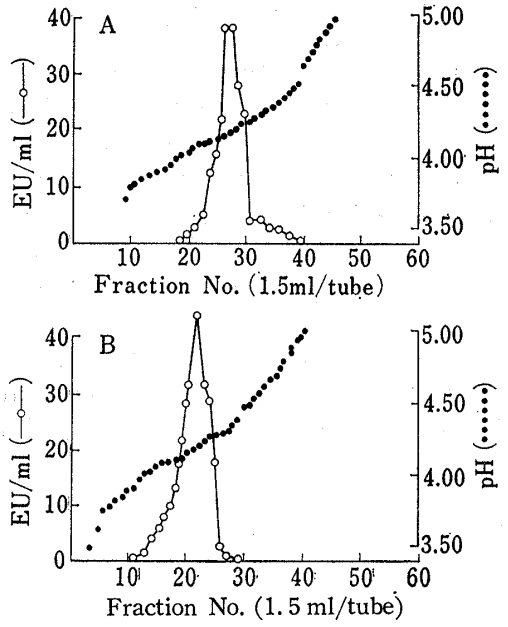


Fig. 5. Isoelectric Focusing of Asialo Hog Pancreatic Kallikreins A and B

A, asialo-kallikrein A (3.0 mg); B, asialo-kallikrein B (3.5 mg). Asialo-kallikreins A and B were prepared by incubation with 0.1 U of neuraminidase for 24 hr at 37° in 0.01 M sodium acetate, pH 6.5.

TABLE II. Carbohydrate Compositions of Hog Pancreatic Kallikreins (residues/mol of kallikrein)

	Fuc	Man	Gal	GlcNAc	NANA	pI	Total sugar
Kallikrein A	0.80	2.20	0.76	2.69	0.24	—	4.56%
B	2.44	3.92	1.78	4.63	0.47	—	8.40%
A-II	1.47	1.36	1.38	2.77	0.57	4.13	5.26%
A-III	2.35	3.41	0.19		0.16	4.24	
B-III	2.93	1.71	1.90	5.30	1.77	4.13	9.19%
B-IV	3.14	1.52	1.35	3.96	0.95	4.19	7.10%
B-V	3.21	4.14	0.48		0.33	4.29	

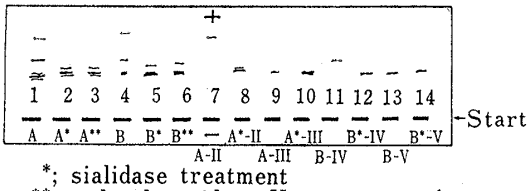
The values were calculated on the basis that the molecular weights of kallikreins A and B are 26800 and 28600, respectively.[9a,b]

Neutral sugars were estimated by gas chromatography (Shimadzu CG 6A; column: 3% OV-225) according to the procedures of Spiro.[19] Glucosamine was estimated with an amino acid analyzer (Hitachi automatic liquid chromatograph, model 0.34; column, Hitachi custum ion exchange resin #2611). Neuraminic acid was determined by the periodate-resorcinol method described by Jourdian *et al.*[20]

and that of Fiedler *et al.*[4b,9b,22] Kallikrein B also contained twice as much N-acetylglucosamine (GlcNAc) and neuraminic acid (NANA) as kallikrein A. In the micro-heterogeneous forms of kallikrein B (B-III and B-IV), a little less than twice as much total sugar was observed as compared with those of kallikrein A (A-II). The fucose contents of kallikreins A-II and A-III were roughly equal (1 or 2 residue(s)/mol of kallikrein) and those of kallikreins B-IV and B-V were also equal (3 residues/mol of kallikrein). As for the micro-heterogeneous forms of kallikreins A and B, the more acidic the form, the higher the contents of both neuraminic acid and galactose.

Preparative and Gel Plate Isoelectric Focusing of Sialo- and Asialo-Kallikreins A and B

Fig. 5 shows the results of preparative isoelectric focusing of the asialo-kallikreins A and B. The micro-heterogeneous forms of the sialo-(intact) kallikreins A and B (Fig. 4-A and -B) largely disappeared in the asialo-kallikreins A and B (Fig. 5-A and -B), and essentially a single peak was observed in each case. The isoelectric points of these two peaks were very similar. Namely, as shown in Fig. 5-A and -B, the pI's of asialo-kallikreins A and B were 4.19 and 4.21, respectively. Similar observations were also obtained by gel plate isoelectric focusing, *i.e.*, after the treatment with neuraminidase more acidic micro-heterogeneous forms of kallikreins A and B were converted to less acidic ones (Fig. 6-2 and -5), thorugh unidentified minor protein bands also appeared. The micro-heterogeneous forms of kallikreins A (A-II and A-III) and B (B-IV and B-V) each gave a single protein band (Fig. 6-7, -9, -11 and -13, respectively). After treatment with neuraminidase, A-II and B-IV showed one protein band shifted to the cathode side (Fig. 6-8 and -12), while in the case of A-III and B-V the bands were not shifted and almost no change was observed (Fig. 6-10 and -14). These results suggested that the minor protein band newly formed A-II and B-IV after the treatment with neuraminidase might be neuraminic acid-free proteins. There was almost no change of the migration positions in the case of the treated A-III and B-V and this may reflect quite small (or zero) constants of neuraminic acid in the intact forms A-III and B-V (Table II). Treatment with endoglycosidase H had no effect on the positions of the bands of asialo-kallikreins A and B (Fig. 6-3 and -6).



*; sialidase treatment
**; endo-glycosidase H treatment after sialidase

Fig. 6. Thin Layer Gel Isoelectric Focusing of Kallikreins

The amount of kallikreins applied was 10—20 μg on 6% (w/v) polyacrylamide gel (0.2×11.0×24.5 cm); focusing was carried out for 3 hr at 25 W (constant) at 4°. Staining was done with 0.1% (w/v) Coomassie brilliant blue R-250.

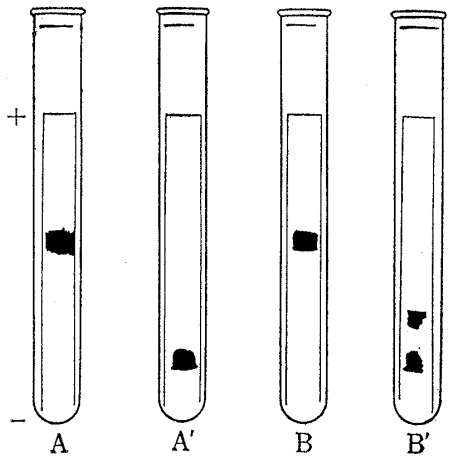


Fig. 7. SDS–Polyacrylamide Gel Electrophoretrams of Kallikreins A and B before and after Treatment with 2-Mercaptoethanol

Samples (60 μg) were applied to the gels, and after electrophoresis, the gels were stained with PAS reagent as described by Zacharius *et al.*[23]
A, B, intact kallikreins A and B, respectively;
A', B', reduced kallikreins A and B, respectively.

SDS-Polyacrylamide Gel Electrophoresis of Kallikreins A and B

Fig. 7 shows the 1% (w/v) SDS-polyacrylamide gel electrophoretograms of kallikreins A and B treated or not treated with 0.04% (v/v) 2-mercaptoethanol in 0.01 M Tris-HCl buffer, pH 7.4, for 30 min at 37°. Gels were stained for sugars with PAS reagent. Kallikreins A and B each showed one hand. In contrast, reduced kallikreins A and B showed one (A′ in Fig. 7) and two (B′ in Fig. 7) bands, respectively. On the other hand, both reduced kallikreins exhibited two bands when gels were stained for proteins with Coomassie brilliant blue R-250. The protein band detected with the reduced kallikrein A (A′ in Fig. 7) and the faster-migrating band of the reduced kallikrein B (one of B′ in Fig. 7) were identical with the bands stained for sugars with PAS reagent. These results suggested that kallikreins A and B both consist of two polypeptide chains linked by a disulfide bond(s), and that one of the chains of kallikrein A has no carbohydrate moiety, while the two polypeptide chains of kallikrein B both contain carbohydrate.

Possible Role of the Neuraminic Acid Residues in the Hetero- and Micro-Heterogeneous Forms of Kallikreins A and B

Removal of the sialyl residues from kallikreins A and B caused no obvious change in their enzymatic properties. The K_m values of the intact heterogeneous kallikreins A and B for Bz-Arg-OEt were 0.140 and 0.096 mM, respectively, and values of 0.099—0.150 mM were observed for micro-heterogeneous forms derived from kallikreins A and B (A-II, A-III, B-IV and B-V in Table III). The K_m values of asialo-kallikreins A and B were 0.110 and 0.150 mM, respectively. Therefore, the K_m values of these kallikreins for Bz-Arg-OEt were in the range of 0.096—0.150 mM, and the sialyl residues seemed not to affect K_m.

TABLE III. K_m Values of Sialo- and Asialo-Kallikreins for Bz–Arg–OEt

Kallikrein		K_m (mM)
Sialo–Kallikrein	A	0.140
	B	0.096
	A-II	0.110
	A-III	0.099
	B-IV	0.120
	B-V	0.150
Asialo–Kallikrein	A	0.110
	B	0.105

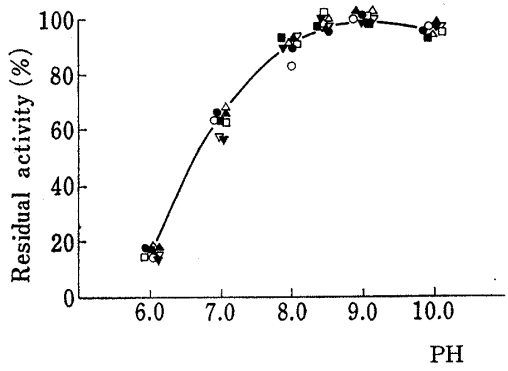


Fig. 8. pH Optima of Sialo- and Asialo–Kallikreins

△, □, ○, intact (sialo) kallikreins A, A-II and A-III, respectively; ▲, ■, ●, intact (sialo) kallikreins B, B-IV and B-V, respectively; ▽, ▼, asialo-kallikreins A and B, respectively.

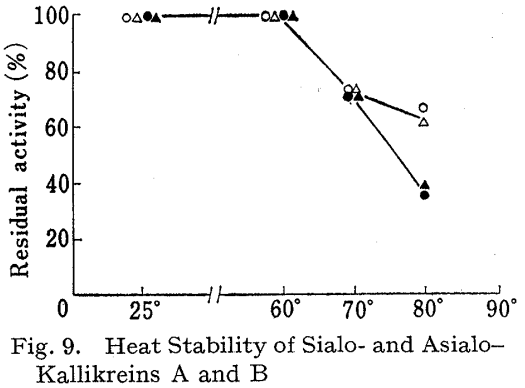


Fig. 9. Heat Stability of Sialo- and Asialo–Kallikreins A and B

○, △, sialo-kallikreins A and B, respectively; ●, ▲, asialo-kallikreins A and B. Intact (sialo) and asialo-kallikreins A and B (1.0 EU) in 0.3 ml of 0.1 M Tris–HCl buffer, pH 8.0, were heated at the indicated temperature for 1 hr. The residual esterolytic activities toward Bz–Arg–OEt were measured.

The optimum pH's of kallikreins A, B, A-II, A-III, B-IV, B-V and asialo-kallikreins A and B for Bz-Arg-OEt hydrolysis were all about 8.5 (Fig. 8).

Fig. 9 shows the thermal stabilities of the sialo- and asialo-kallikreins A and B. No significant differences of thermal stability were observed between the sialo- and asialo-enzymes under 70°. However, sialo-kallikreins A and B were more stable than asialo-kallikreins A and B when they were treated at 80° for 1 hr.

According to Fiedler *et al.*,[4a] the amino acid compositions of kallikreins A and B are identical except for an excess of 3 amide ammonia residues in kallikreins B. As regards sugar content, our previous[10] and present kallikrein B contained about twice as much neutral hexose and glucosamine as kallikrein A. This difference in amounts of sugars was stated by Fiedler *et al.*[4a,9b,22] to be sufficient to allow the separation of these kallikreins A and B by anion-exchanger chromatography and electrophoresis. They did not determine the neuraminic acid content because they isolated these kallikreins after neuraminidase treatment. As regards the neuraminic acid contents of micro-heterogeneous forms A-II, A-III, B-III, B-IV and B-V, the more acidic micro-heterogeneous forms have lower pI values (Table II). Moreover, neuraminidase-treated kallikreins A and B each showed a single active peak on isoelectric focusing (Fig. 5-A and -B). Judging from these observations, we concluded that the differences in acidity at the micro-heterogeneous level (but not at the heterogeneous level) are due to differences in the amounts of neuraminic acid in the molecules. In our previous paper,[10] carbohydrate determination was carried out for kallikreins B-I (pI 3.80 and 3.82), B-2 (pI 3.92 and 4.01) and B-3 (4.12 and 4.18), which were each mixtures of two micro-heterogeneous forms of kallikrein B having slightly different pI's. In the present paper, the carbohydrate compositions of the main micro-heterogeneous forms of kallikreins A and B (A-II, A-III, B-III, B-IV and B-V in Fig. 4) were separately determined in detail (Table II) in order to clarify the relationship between the micro-heterogeneous forms and the carbohydrate contents. The observations on the neuraminidase-treated kallikreins A and B indicate that the neuraminic acid residue may not be related to the enzymatic activities, but may influence the thermal stabilities of the enzymes. Therefore, the carbohydrate moiety of the kallikrein might contribute to maintenance of the three-dimensional structure of the protein, but not to the enzymatic activity. Similar results were reported in the cases of sulfatase[24] and aminopeptidase.[25] With regard to the roles of sugars in protein chemistry, the fact that neuraminidase treatment causes conformational changes had already been demonstrated in the case of human prostate acid phosphatase.[26] Furthermore, in the case of some glycoenzymes, such as human intestinal maltase and sucrase, changes of antigenicity upon removal of carbohydrate moieties were also reported by Kelly and Alpers.[27] Besides these observations, the effects of neuraminic acid on the catabolism of glycoproteins have been extensively investigated.[28] Further studies of the roles or functions of the carbohydrate moieties in kallikreins seem desirable, not only with hog pancreatic kallikrein but also with other kallikreins, especially those of human origin.

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