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Evidence for the Presence of Two Kininogens in Porcine Plasma and the Isolation of High-Molecular-Weight Kininogen[1)]

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When the pseudoglobulin fraction of porcine plasma was gel-filtered through a column of Sephadex G-150, two fractions, from which kinin was released on incubation with trypsin [EC 3.4.21.4], were observed. This indicates the presence of two kininogens with different molecular weights in the plasma. The larger-molecular-weight kininogen fraction had the ability to correct the abnormal clotting time of human high-molecular-weight (HMW) kininogen-deficient plasma, Fitzgerald plasma. The smaller-molecular-weight kininogen fraction did not show this ability. Thus, the fraction showing the ability to correct the abnormal clotting time of Fitzgerald plasma was considered to contain a kininogen corresponding to that purified as an HMW kininogen from bovine, human, horse and rat plasmas. The other fraction may contain porcine low-molecular-weight (LMW) kininogen.

The porcine HMW kininogen was isolated by a modification of the purification method for horse HMW kininogen. The ability of the isolated HMW kininogen obtained to correct the abnormal clotting time of Fitzgerald plasma was in proportion to the amount of the preparation used. When this HMW kininogen preparation was incubated with purified porcine plasma kallikrein [EC 3.4.21.8], the release of bradykinin was observed.

Keywords——kininogen; high-molecular-weight kininogen; porcine plasma; isolation; blood coagulation

Introduction

Protein components related to the kallikrein–kinin system have been isolated from several mammalian plasmas,[2)] and it is known that two precursor proteins for kinins, high-molecular-weight (HMW) and low-molecular-weight (LMW) kininogens, are widely present in mammalian plasmas.[3)] This HMW kininogen is a natural substrate for plasma kallikrein and has been recognized as a co-factor which accelerates the initiation reaction for the blood coagulation cascade system. Furthermore, it was reported that HMW kininogen has the ability to inhibit thiol-proteinases.[4)]

HMW kininogens have been purified from bovine,[5)] human,[6,7)] horse[8)] and rat[9)] plasmas. However, no report has appeared on the purification of porcine HMW and LMW kininogens. Even the existence of two kinds of kininogens in porcine plasma has not been clarified, and the kinin-releasing mechanism of the kallikrein–kinin system in porcine plasma is also obscure. Therefore, we tried to isolate the protein components of the kallikrein–kinin system in porcine plasma, and purified porcine plasma prekallikrein.[10)] In this paper, evidence for the presence of two kininogens in porcine plasma is presented. The porcine pseudoglobulin fraction was used as the starting material, and the gel-filtration method with a column of Sephadex G-150 was used. The larger-molecular-weight kininogen fraction, which may contain HMW kininogen, had the ability to correct the abnormal clotting time of HMW kininogen-deficient human plasma (Fitzgerald plasma). Also, kinin was released from this fraction through the action of trypsin. Thus, we tried to isolate porcine HMW kininogen by modification of the

purification procedures for the HMW kininogen from bovine[5] and horse[8] plasmas. When we were purifying porcine plasma prekallikrein, it was found that the factor XII content of porcine plasma is very much higher than that of other mammalian plasmas, and even when a minute amount of this factor XII is activated during the purification procedures for prekallikrein or HMW kininogen, the activated factor XII converts prekallikrein to kallikrein. Consequently, during the purification procedures, kallikrein thus activated released bradykinin from HMW kininogen, and so we usually obtained kinin-free kininogen. Thus, the usual purification procedures for bovine[5] or horse[8] HMW kininogen could not be easily applied. For the purification of the porcine HMW kininogen, a method for completely preventing the appearance of activated factor XII or for inhibiting the slight amount of kallikrein activity that appeared during the isolation procedure was required. For this, the addition of Polybrene®, benzamidine or diisopropylfluorophosphate (DFP) at every purification step was necessary. This approach allowed us to isolate HMW kininogen from porcine plasma.

Experimental

Materials——Diethyl aminoethyl (DEAE)-Sephadex A-50, carboxymethyl (CM)-Sephadex C-50, Sephadex G-150 and Sephadex G-50 were purchased from Pharmacia Fine Chemicals, Sweden. Synthetic bradykinin, kallidin, Met–Lys–bradykinin and *N*-α-tosyl-L-arginine methylester (TAME) were obtained from the Peptide Institute Inc., Japan. Fitzgerald human plasma was obtained from George King Bio-Medical, U.S.A. Trypsin (twice crystallized), rabbit brain cephalin and a kit of standard protein markers (MW-SDS-200) for the determination of molecular weight by sodium dodecyl sulfate (SDS)-gel electrophoresis were purchased from Sigma Chemicals, Co., U.S.A. *p*-Aminobenzamidine-ε-aminocaproic acid-Sepharose 4B was synthesized in our laboratory according to the method of Holleman and Weiss.[11] The sources of other materials were as follows: DFP from Katayama Kagaku Kogyo Co., Ltd., Japan; Trasylol® from Bayer, West Germany; benzamidine hydrochloride from Tokyo Kasei Kogyo Co., Ltd., Japan; and Polybrene® (hexadimethrine bromide) from Aldrich Chemicals Co., U.S.A.

Porcine Plasma for Chromatography——Fresh porcine blood containing 3.8% sodium citrate was collected in polyethylene bottles at a slaughterhouse, and centrifuged at 3000 rpm for 30 min at 20 °C. The plasma obtained was supplemented with Polybrene® at a concentration of 400 mg/l and benzamidine at a final concentration of 5 mM, and stocked at −80 °C until use.

Pseudoglobulin Fraction of Porcine Plasma——Porcine plasma (40 ml) was mixed with a saturated ammonium sulfate solution to obtain 30% saturation at 4 °C. After being stirred for 30 min, the suspension was centrifuged at 5000 rpm for 15 min. The supernatant was collected and mixed with a saturated ammonium sulfate solution to obtain 50% saturation. The suspension was stirred for 1 h, then centrifuged at 9000 rpm for 20 min. The pseudoglobulin fraction thus obtained was dissolved in 10 ml of 0.02 M phosphate buffer, pH 7.5, containing 0.13 M NaCl, followed by dialysis overnight against 5 l of the same buffer at 4 °C.

Preparation of Porcine Plasma Kallikrein——The plasma kallikrein fraction, which was spontaneously activated during the purification of porcine plasma prekallikrein,[10] was used as the starting material. This kallikrein fraction was applied to a column (1.5 × 6 cm) of *p*-aminobenzamidine-ε-aminocaproic acid-Sepharose 4B equilibrated with 0.02 M Tris–HCl buffer, pH 8.5, and the column was washed with 200 ml of 0.02 M Tris–HCl buffer, pH 8.5, containing 0.8 M NaCl. Elution was performed with a linear concentration gradient of benzamidine formed from 200 ml each of 0 M and 0.1 M benzamidine in 0.02 M Tris–HCl buffer, pH 8.5, containing 0.8 M NaCl. The kallikrein fraction obtained from the above chromatography was dialyzed against 10 l of 0.02 M Tris–HCl buffer, pH 8.5, at 4 °C overnight, and then applied to a column (1.2 × 12 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. Kallikrein was eluted with a linear concentration gradient formed from 200 ml each of 0.02 M Tris–HCl buffer, pH 8.5, and the same buffer containing 0.4 M NaCl. The kallikrein fraction was detected on the basis of the kinin-releasing activity,[12] and the TAME-hydrolytic activity.[13] The specific activity of the porcine plasma kallikrein thus obtained was 55.2 μmol of TAME per min (TAME unit) per mg of protein. The preparation gave a single band on polyacrylamide gel disc electrophoresis according to the method of Davis.[14]

Bioassay of Kinin——When benzamidine had been added to the elution buffer for ion-exchange chromatography, aliquots of each eluate were dialyzed against 5 l of 0.02 M Tris–HCl buffer, pH 8.0, containing 0.13 M NaCl at 4 °C for 6 h. Then aliquots of the kininogen fraction were incubated with 50 μg of trypsin or 0.4 TAME unit/ml of the purified porcine plasma kallikrein at 37 °C for 10 min in the presence of *o*-phenanthroline at a final concentration of 3 mM. The reaction was terminated by the addition of Trasylol® (900 K.I.U.). Kinin liberated was estimated in terms of its activity for the contraction of guinea-pig ileum, with synthetic bradykinin as a standard.[12] One unit of kininogen was defined as the amount of kininogen producing kinin activity equivalent to that of 1 μg of synthetic

bradykinin. Specific activity was expressed as kininogen units per mg of protein.

Coagulant Assay for HMW Kininogen——The assay of kaolin-activated partial thromboplastin time was carried out according to the method of Matheson *et al.*[15] using Fitzgerald plasma. The mixture containing 50 μl each of the HMW kininogen fraction, kaolin–cephalin reagent and Fitzgerald plasma was incubated for 30 min at 37 °C, and after the addition of 50 μl of 0.05 M $CaCl_2$, the clotting time was measured at 37 °C. The coagulant activity was expressed as the clotting time.

Identification of Kinins——High-performance liquid chromatography (HPLC) for the identification of kinins was performed according to the modified method of Geiger *et al.*[16] with the use of a Waters model 441 apparatus. The kinins were identified by the comparison of each retention time with that of standard bradykinin, kallidin or Met–Lys–bradykinin.

Electrophoresis——Disc electrophoresis was carried out on a column (5 × 50 mm) of 7% polyacrylamide gel, using Tris–glycine buffer, pH 8.3[14] SDS-gel electrophoresis was carried out by the method of Weber and Osborn.[17] Fifteen μg of protein was used. The gels were stained with 0.25% Coomassie brilliant blue R-250 and destained with a mixture of 7.5% acetic acid and 5% methanol. The molecular weight of proteins was estimated from their relative mobilities on 7% gel, in comparison with those of standard proteins reduced with 2-mercaptoethanol.

Protein Concentration——Protein concentrations were determined by measurement of the absorbance at 280 nm, assuming that an absorption value of 1.0 equals 1 mg/ml.

Analysis of Amino Acid Composition——The amino acid composition of the kinin released from the HMW kininogen by plasma kallikrein was determined with an amino acid analyzer, Hitachi model 835, after hydrolysis in 0.2 ml of constant-boiling 5.7 N HCl at 105 °C for 24 h.

Results and Discussion

Evidence for the Existence of Two Kininogens in Porcine Plasma

The porcine pseudoglobulin fraction was passed through a gel-filtration column (3.5 × 90 cm) of Sephadex G-150 equilibrated with 0.02 M phosphate buffer, pH 7.5, containing 0.13 M NaCl. When aliquots of each fraction were incubated with trypsin, peaks of kinin activity were detected in two fractions, fraction numbers 65—100 and 101—115. However, kinin activity due to the action of the purified porcine plasma kallikrein was not detected in any of the incubation mixtures (Fig. 1). So, it was not clarified which of the above two kininogen fractions contained HMW kininogen.

It is known that human or bovine HMW kininogen,[15,18-20] but not the corresponding LMW kininogen,[15,19] has the ability to correct the abnormal clotting time of human HMW kininogen-deficient plasma. Therefore, the above ability for human HMW kininogen-deficient plasma (Fitzgerald plasma) was tested using fraction numbers 79 and 106. In the case of fraction number 79, the clotting time of Fitzgerald plasma was 160 s, but in the case of fraction number 106 and physiological saline, the clotting times of the plasma were 790 and 780 s, respectively. This indicates that porcine HMW kininogen is contained in the faster-

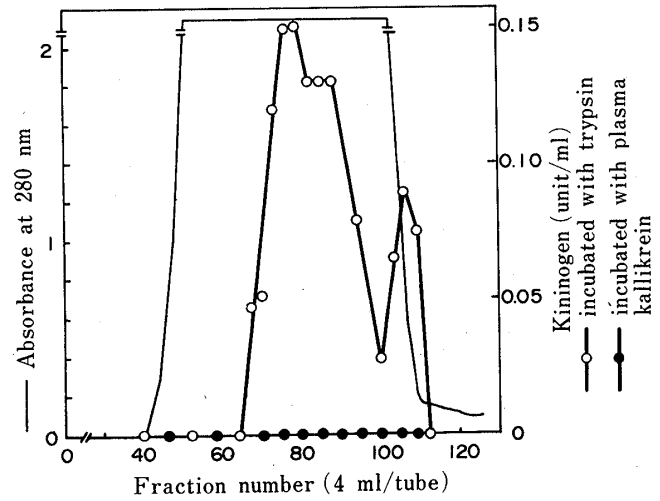

Fig. 1. Gel-Filtration of the Pseudoglobulin Fraction from Porcine Plasma

The porcine pseudoglobulin fraction was applied to a column of Sephadex G-150 and elution was carried out at a flow rate of 20 ml per h at 4 °C.

eluted kininogen fraction. The slower-eluted fraction, from which kinin was released through the action of trypsin, did not have the ability to correct the abnormal clotting time of Fitzgerald plasma. Thus, it is considered that porcine LMW kininogen is contained in this fraction. These results indicate that two kininogens, HMW and LMW kininogens, are also present in porcine plasma, and the content of HMW kininogen was higher than that of LMW kininogen.

As one reason why kinin activity due to the action of the purified plasma kallikrein was not detected (Fig. 1), it is considered that a plasma kallikrein inhibitor coexists in the HMW kininogen fraction in Fig. 1 (data not shown).

Isolation of Porcine HMW Kininogen

Porcine plasma was centrifuged at 5000 rpm for 10 min at 20 °C to remove the precipitate when the frozen plasma was used as a starting material. All of the columns and fraction-collecting tubes were silicone-coated before use.

Plasma (1 l) was applied to a column (7.5 × 13.5 cm) of DEAE-Sephadex A-50 equilibrated with 0.02 M Tris–HCl buffer, pH 8.0, containing 0.1 M NaCl, 5 mM benzamidine and Polybrene® (400 mg/l) at room temperature. The column was then washed with 1 l of 0.02 M Tris–HCl buffer, pH 8.0, containing 0.2 M NaCl, 5 mM benzamidine and Polybrene® (400 mg/l). The protein was eluted with 1 l of 0.05 M acetate buffer, pH 6.0, containing 0.8 M NaCl, 5 mM benzamidine and Polybrene® (400 mg/l). Each 100 ml fraction was collected in a plastic bottle at room temperature. Kininogen, from which kinin was released on incubation with trypsin, was found in the fractions eluted with the buffer containing 0.8 M NaCl. The fractions containing the kininogen were combined and the pooled kininogen fraction was supplemented with 1 ml of 1 M DFP. The solution was allowed to stand overnight at 4 °C, then diluted with about 1.5 volumes of cold water containing 5 mM benzamidine and Polybrene® (400 mg/l). The pH was adjusted to pH 6.0 with 10% acetic acid. After addition of 1 ml of 0.1 M DFP, the solution was allowed to stand for 1 h at 4 °C, and then applied to a column (7.5 × 4.0 cm) of CM-Sephadex C-50 equilibrated with 0.05 M acetate buffer, pH 6.0, containing 0.3 M NaCl, 5 mM benzamidine and Polybrene® (400 mg/l) at 4 °C. Then, the column was washed with 300 ml of the same buffer, and the protein was eluted with 300 ml each of the same buffer containing 0.45 M NaCl, 0.6 M NaCl and 0.7 M NaCl, respectively. Each 100 ml fraction was collected in a plastic bottle in a cold room. Kininogen was eluted in the fractions from 101 to 700 ml. To the kininogen fraction thus obtained, 1 ml of 0.1 M DFP was added, and the solution was allowed to stand overnight at 4 °C and dialyzed against 20 l of 0.02 M Tris–HCl buffer, pH 8.0, containing Polybrene® (400 mg/l) and 0.13 M NaCl overnight. Then 1 ml of 0.1 M DFP was added to the dialysate, and the solution was allowed to stand for 1 h at 4 °C. It was then applied to a column (1.5 × 4.5 cm) of *p*-aminobenzamidine-ε-aminocaproic acid-Sepharose 4B equilibrated with 0.02 M Tris–HCl buffer, pH 8.0, containing 0.13 M NaCl and Polybrene® (40 mg/l) at 4 °C. Kininogen was only eluted in the breakthrough fraction. To the kininogen fraction obtained in the previous step, 1 ml of 0.1M DFP was added, and the solution was allowed to stand overnight at 4 °C and dialyzed against 10 l of 0.05 M acetate buffer, pH 6.0, containing 0.2 M NaCl and Polybrene® (40 mg/l) overnight at 4 °C. Then 1 ml of 0.1 M DFP was added to the dialysate. After the solution had been allowed to stand for 1 h at 4 °C, it was applied to a column (3.5 × 5.0 cm) of CM-Sephadex C-50 equilibrated with 0.05 M acetate buffer, pH 6.0, containing 0.2 M NaCl and Polybrene® (40 mg/l) at 4 °C. Kininogen was eluted with a linear concentration gradient formed from 250 ml each of 0.05 M acetate buffer, pH 6.0, containing 0.2 M NaCl and Polybrene® (40 mg/l), and the same buffer containing 0.8 M NaCl and Polybrene® (40 mg/l). The kininogen was found in the fractions eluted with the buffer containing 0.45 M NaCl. To the kininogen fraction obtained in the previous step, 1 ml of 0.1 M DFP was added, and the solution was

allowed to stand overnight at 4 °C and dialyzed against 10 l of 0.02 M Tris–HCl buffer, pH 8.5, containing 0.1 M NaCl overnight. Then 1 ml of 0.1 M DFP was added to the dialysate. The solution was a allowed to stand for 1 h at 4 °C, and applied to a column (2.5 × 4.5 cm) of DEAE-Sephadex A-50 equilibrated with 0.02 M Tris–HCl buffer, pH 8.5, containing 0.1 M NaCl at 4 °C. The protein was eluted with a linear concentration gradient of 0.1 to 0.6 M NaCl in the buffer (each 200 ml). As shown in Fig. 2, the kinin activity was found in the major protein peak after incubation of each fraction with the purified porcine plasma kallikrein. The fractions from tubes 24—41, indicated by the arrow in Fig. 2, were pooled.

A summary of the procedures for the isolation of porcine HMW kininogen is shown in Table I. The yield of HMW kininogen was about 6.1 mg from 1 l of porcine plasma. The specific activity of the final preparation was 12.6, this value being lower than that for the

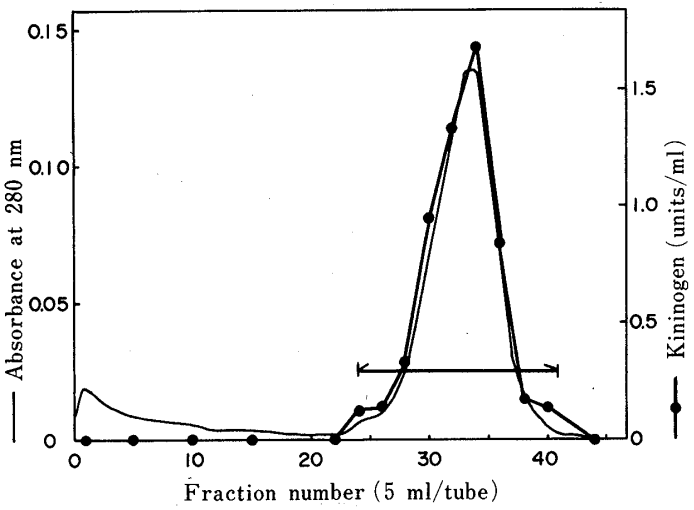

Fig. 2. Second DEAE-Sephadex A-50 Column Chromatography of the Porcine HMW Kininogen Fraction

The porcine HMW kininogen fraction obtained from the 2nd CM-Sephadex C-50 chromatography was chromatographed on a column of DEAE-Sephadex A-50. The fractions indicated by the arrow were pooled.

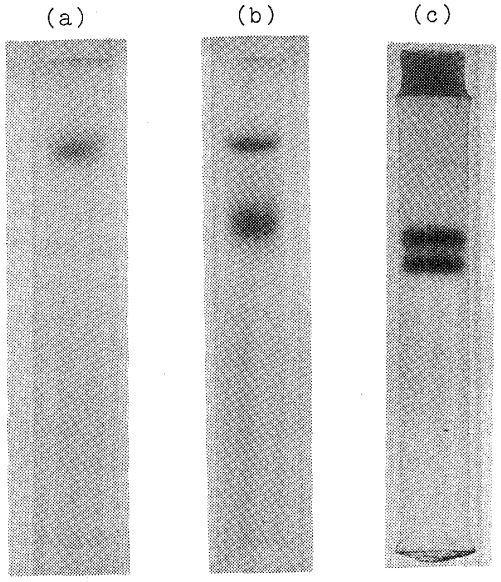

Fig. 3. Polyacrylamide Gel Electrophoresis of the Isolated Porcine HMW Kininogen

The procedures and conditions used are described in the text. a) Non-reduced porcine HMW kininogen in SDS-gel, phosphate buffer, pH 7.2; b) reduced porcine HMW kininogen in SDS-gel, phosphate buffer, pH 7.2; c) porcine HMW kininogen with Tris–glycine, pH 8.3.

TABLE I. Summary of the Purification Procedures for Porcine HMW Kininogen

Procedures	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
1st DEAE-Sephadex A-50 column chromatography	2700	364	0.13	100
1st CM-Sephadex C-50 column chromatography	79	149	1.89	40.9
Affinity chromatography on *p*-aminobenzamidine-ε-amino-caproic acid-Sepharose 4B	63	139	2.21	38.2
2nd CM-Sephadex C-50 column chromatography	7.5	80.5	10.77	22.1
2nd DEAE-Sephadex A-50 column chromatography	6.1	76.5	12.59	21.0

HMW kininogen from bovine[5] or horse[8] plasma. The specific activities of the bovine and horse HMW kininogens were 14.2 and 20.2, respectively.

During the isolation procedures for porcine HMW kininogen mentioned above, the following precautions were necessary: 1) addition of DFP at all isolation steps to inhibit kallikrein activity, 2) addition of a large amount of Polybrene® at an early step to prevent activation of factor XII, and 3) removal of a small amount of kallikrein or prekallikrein from the HMW kininogen fraction by column chromatography on *p*-aminobenzamidine-ε-aminocaproic acid-Sepharose 4B.

In the case of the bovine kininogens, the LMW kininogen was eluted in the breakthrough fraction of the 1st CM-Sephadex C-50 chromatography at pH 6.0, so the HMW and LMW kininogens could be easily separated.[5] However, in our case, kinin activity was not detected in the breakthrough fraction of this chromatography. This result suggests that there is no LMW kininogen in the fraction obtained from the 1st DEAE-Sephadex A-50 chromatography. In fact, kinin activity was also detected in the breakthrough fraction of the 1st DEAE-Sephadex A-50 chromatography, so the HMW and LMW kininogens may be separated at this step, although we have not yet been able to isolate LMW kininogen from this fraction.

Purity

The purity of the final preparation was examined by electrophoresis on polyacrylamide gel using Tris–glycine, pH 8.3,[14] and SDS-polyacrylamide gel electrophoresis in the presence or absence of 2-mercaptoethanol.[17] As shown in Fig. 3, the preparation obtained gave two bands on polyacrylamide gel electrophoresis at pH 8.3, though it gave a single band on SDS-polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol. The molecular weight of porcine HMW kininogen was estimated to be about 110000 from the electrophoresis. When the preparation was treated with 10% 2-mercaptoethanol and then subjected to SDS-polyacrylamide gel electrophoresis, a new band showing a faster mobility was detected in addition to a band which showed the same mobility as the band of the preparation not treated with 2-mercaptoethanol. The molecular weight of this protein was estimated to be about 60000 from the electrophoresis. We are attempting to isolate each protein component in the final preparation, but it is very difficult to purify the protein components by ion-exchange chromatographies. When the preparation was subjected to isoelectric focusing, each protein component was partially separated, judging from the results of polyacrylamide gel electrophoresis, and kinin was released from each component on incubation with trypsin or plasma kallikrein. These results indicate that both of the above protein components are HMW kininogen, and the preparation obtained contains heterogeneous molecules of porcine HMW kininogen. By analogy with the case of bovine or horse HMW kininogens,[8,21] one of the porcine HMW kininogens thus prepared may consist of a single polypeptide chain with a molecular weight of 110000 and the other may consist of two polypeptide chains. The HMW kininogen consisting of two polypeptide chains seems to be formed from the intact kininogen during the purification procedure, although these protein components were always contained in the preparation. The isoelectric point of porcine HMW kininogen was determined to be about 4.67 by the isoelectric focusing method.

The preparation did not contain plasma kallikrein, since no spontaneous liberation of kinin was observed on incubation with 100 μg of the preparation at 37 °C for 2 h. Plasma kininase did not contaminate this preparation, because the kinin released from the kininogen preparation after incubation with porcine plasma kallikrein at 37 °C for 30 min was not destroyed.

Correction of the Abnormal Clotting Time of the Fitzgerald Plasma by Porcine HMW Kininogen

As shown in Fig. 4, the isolated porcine HMW kininogen shortened the abnormal

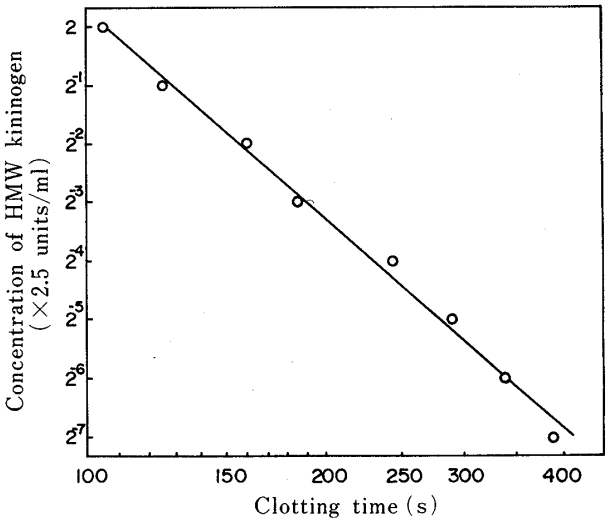

Fig. 4. Correction of the Abnormal Clotting Time of Fitzgerald Plasma by the Isolated Porcine HMW Kininogen

The test samples used were prepared by serial two-fold dilution of the original porcine HMW kininogen solution (about 0.2 mg/ml). The assay conditions used are described in the text.

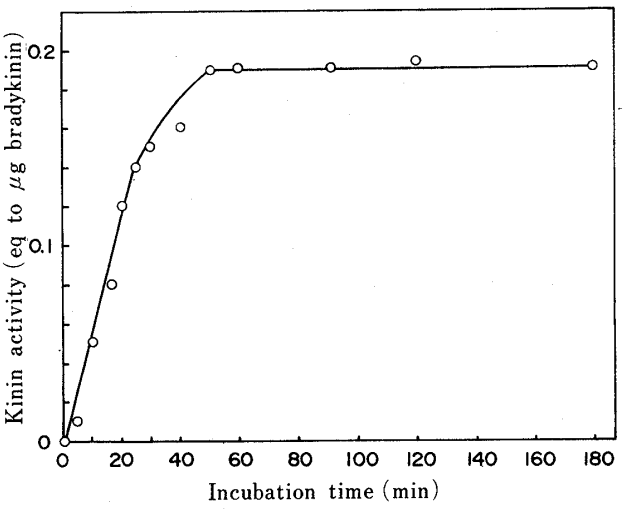

Fig. 5. Liberation of Kinins from Porcine HMW Kininogen by Porcine Plasma Kallikrein

The reaction mixture, containing 50 μl of the isolated porcine HMW kininogen (5 units/ml), 5 μl of *o*-phenanthroline (30 mM), and 1 μl of the purified porcine plasma kallikrein (0.4 TAME unit/ml), was incubated at 37 °C. At the indicated times, a 50 μl aliquot was pipetted into an organ bath (20 ml) to determine the kinin activity.

clotting time of Fitzgerald plasma in proportion to the amount of the preparation used. This indicates that porcine HMW kininogen has the ability to correct the abnormal clotting time of Fitzgerald plasma.

Liberation and Identification of Kinin from Porcine HMW Kininogen through the Action of Porcine Plasma Kallikrein

Figure 5 shows the rate of liberation of kinin from the isolated porcine HMW kininogen by purified porcine plasma kallikrein. The maximum release of kinin was seen within 60 min, when an enzyme: substrate ratio of 1 : 2700 (w/w) was used.

To identify the kinin liberated from porcine HMW kininogen by porcine plasma kallikrein, the isolated kininogen solution (31 mg) was incubated with 0.4 TAME unit of the purified porcine plasma kallikrein at 37 °C for 24 h. The incubated mixture was gel-filtered through a column (2.6 × 50 cm) of Sephadex G-50 equilibrated with 5% acetic acid. The kinin fraction thus obtained was lyophilized and dissolved in 1 ml of distilled water. When the solution was subjected to HPLC, the elution profile of the kinin resembled that of bradykinin (Fig. 6). The kinin fraction eluted from the column was collected and lyophilized. An aqueous solution of the lyophilized material was mixed with standard bradykinin solution, and the solution was subjected to HPLC. Only one peak of bradykinin was detected in this chromatogram. Furthermore, from the analysis of the amino acid composition of the kinin fraction obtained from the chromatography (Table II), the kinin liberated from porcine HMW kininogen by the purified porcine plasma kallikrein was identified as bradykinin.

Molecular Changes of Porcine HMW Kininogen Accompanying the Release of Bradykinin by Porcine Plasma Kallikrein

A mixture containing the isolated porcine HMW kininogen and purified porcine plasma kallikrein was incubated at 37 °C for the indicated times under the same conditions as described above (Fig. 5), and each incubation mixture was subjected to polyacrylamide gel

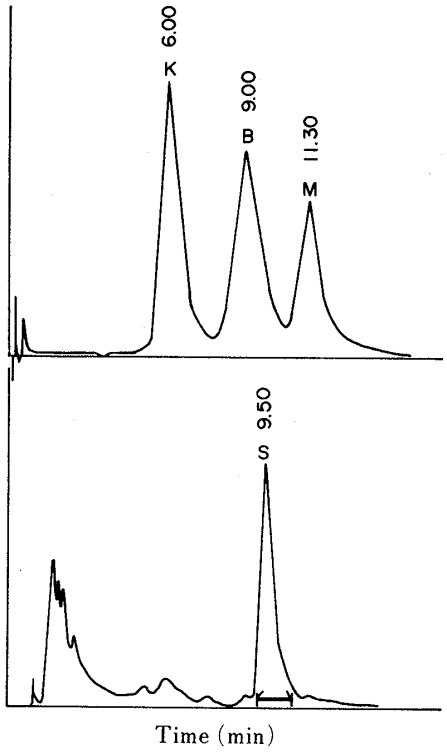

Fig. 6. HPLC of Kinins

The procedures and conditions used were as follows; column: μ Bondapak C_{18} equilibrated with 20% acetonitrile in 0.1 M sodium phosphate buffer, pH 7.0; flow rate: 1.5 ml per min; detection wavelength: 214 nm. The chromatography was carried out at room temperature. B, bradykinin; K, kallidin; M, Met–Lys–bradykinin; S, liberated from the isolated porcine HMW kininogen by purified porcine plasma kallikrein.

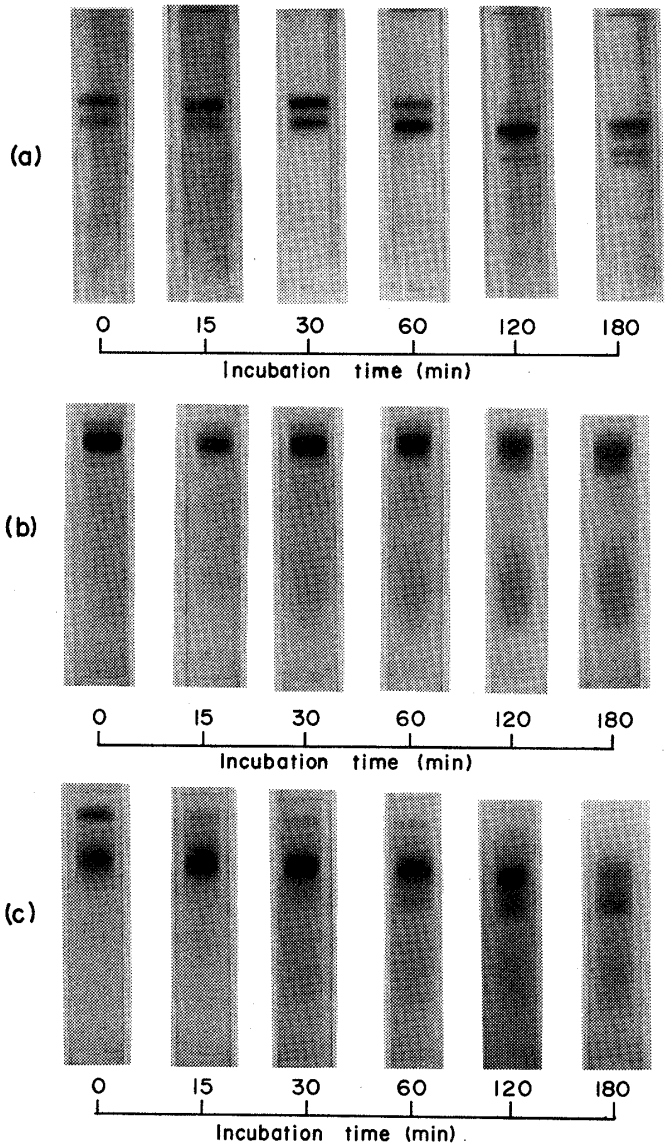

Fig. 7. Polyacrylamide Gel Electrophoresis of the Incubation Mixture of Porcine HMW Kininogen and Porcine Plasma Kallikrein

After 40 μl of the isolated porcine HMW kininogen (5 units/ml) was incubated with 1 μl of the purified porcine plasma kallikrein (0.3 TAME unit/ml) at 37 °C for the indicated times, each incubation mixture was electrophoresed on 7% polyacrylamide gel in the presence or absence of SDS. a) Tris–glycine, pH 8.3; b) non-reduced protein in SDS-gel, phosphate buffer, pH 7.2; c) reduced protein in SDS-gel, phosphate buffer, pH 7.2. The migration was from top (cathode) to bottom.

electrophoresis at pH 8.3[14] or SDS-polyacrylamide gel electrophoresis[17] in the presence or absence of 2-mercaptoethanol (Fig. 7). During the course of the incubation of the HMW kininogen and plasma kallikrein, the incubated mixture gave new bands showing a faster mobility than the original bands on polyacrylamide gel electrophoresis at pH 8.3 (Fig. 7a). In the case of SDS-polyacrylamide gel electrophoresis, the band of the incubated mixture showed almost the same mobility as the original band in the absence of 2-mercaptoethanol (Fig. 7b), but the incubated mixture gave new bands showing a faster mobility than the

TABLE II. Amino Acid Composition of Kinin Liberated from Porcine HMW Kininogen through the Action of Porcine Plasma Kallikrein

Amino acid	nmol	Residues per mol[a)]
Ser	0.29	1.00 (1)
Gly	0.35	1.21 (1)
Arg	0.55	1.89 (2)
Pro	0.78	2.70 (3)
Phe	0.58	2.00 (2)

a) The value for phenylalanine was taken as 2 and the most probable integral numbers of residues are indicated in parenthesis.

original band in the presence of 2-mercaptoethanol (Fig. 7c). These results suggest that small polypeptidic fragments in addition to bradykinin are released during the incubation of the HMW kininogen and plasma kallikrein. Detailed studies of the fragmentation of porcine HMW kininogen by porcine plasma kallikrein are in progress and the results will be reported in the near future.

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