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Porcine Pancreatic Prokallikrein. II. Purification and Some Properties of Prokallikrein A[1)]

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The presence of two main components of porcine pancreatic prokallikrein (prokallikreins A and B) was observed on anion-exchange chromatography. One of them, prokallikrein A, was purified by water extraction, followed by a combination of ammonium sulfate fractionation, ion-exchange chromatographies, preparative disc gel electrophoresis and gel filtration in the presence of various protease inhibitors.

Prokallikrein A reacted immunologically with the antibody against kallikrein A obtained from autolyzed porcine pancreas. It migrated slightly more slowly than kallikreins A and B on immunoelectrophoresis.

The molecular weight of prokallikrein A was estimated to be $3.6—3.9 \times 10^4$ by high performance liquid chromatography. On the other hand, those of kallikreins A and B were estimated to be $3.3—3.4 \times 10^4$ by the same method. Thus, it was speculated that at least large fragment would not be liberated from the prokallikrein molecule during the activation by protease(s).

Keywords——kallikrein; kallikrein–kinin system; porcine pancreatic kallikrein; prokallikrein A; glandular kallikrein; immunoelectrophoresis

At present, various glandular kallikreins have been purified from various organs of many species and their properties investigated. However, the physiological and/or pathological significance of the glandular kallikrein–kinin system in the body is still poorly understood. The authors have attempted to isolate prokallikrein from porcine pancreas in order to aid in clarifying the biological significance of the glandular kallikrein–kinin system in the body,[1,2)] since it should be easier to obtain information on the enzyme(s) involved in the activation of glandular prokallikrein in the body or on the mechanism of activation of prokallikrein by using an isolated prokallikrein preparation. So far, several groups have attempted to isolate prokallikrein from the pancreas,[3,4)] urine,[5)] kidney[6)] of mammals but satisfactorily purified or even partially purified preparations have not been obtained in many cases, mainly due to the rapid activation to kallikrein by protease(s) presents in the organs and to unexplained spontaneous activation during the purification procedures as shown in Fiedler's report.[3)] The authors have attempted to suppress activation during the purification of prokallikrein by the use of various protease inhibitors and have succeeded in the partial purification of prokallikrein from porcine pancreas as a stable pro-form preparation.[1,2)] In our previous paper, the presence of two components of prokallikrein was described.[1)] However, these two prokallikrein components were not completely separated with each other. Therefore, in the previous work, we purified the mixture of these two prokallikrein components and investigated the enzyme(s) involved in the activation of the prokallikrein thus obtained.[1)]

In the present work, we attempted to separate the two prokallikrein components. This paper deals with the purification and some properties of one of these components, prokallikrein A, from porcine pancreas.

Materials and Methods

Materials——Diethylaminoethyl (DEAE)-Sepharose CL-6B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Other reagents used were the same as mentioned in our

previous paper.[1]

Esterolytic Activity Assay[1,7]——Esterolytic activity towards N^{α}-benzoyl-L-arginine ethyl ester (BzArgOEt) was photometrically measured as a kallikrein assay and the activity was expressed in esterase units (EU). One EU is the amount of enzyme that can hydrolyze 1 μmol of BzArgOEt per min at 25°C and pH 8.0. The amount of prokallikrein was determined in the same way as in our previous paper, namely the amount of prokallikrein was estimated and expressed as EU after activation with trypsin.[1]

Preparative Disc Gel Electrophoresis——This method was carried out using an apparatus manufactured by Canal Industrial Co. (Rockville, Maryland, U.S.A.), based on the method of Davis.[8]

High Performance Liquid Chromatography (HPLC)——HPLC was carried out using a coupled combination of two Shodex A-803 (8×500 mm) columns and one Shodex A-804 (8×500 mm) column (Showa Denko Co., Tokyo). The order of column connection was Shodex A-803, -803 and -804 columns, from the top.

Preparation of Anti-porcine Pancreatic Kallikrein A Serum——Porcine pancreatic kallikrein A which had been highly purified in our laboratory (1350 KU/mg, 102 EU/E_{280})[9] was used for the preparation of anti-porcine pancreatic kallikrein A serum. Porcine pancreatic kallikrein A solution (1.0 mg/ml), 1.5 ml, was emulsified with an equal volume of complete Freund's adjuvant and this emulsion was injected intracutaneously into the foot pads and the backs of rabbits weighing 2.5 kg. After 17 days, the same emulsion was injected intracutaneously into the foot pads and the backs of the rabbits. The whole blood was collected 13 days after the second injection. The serum was obtained by centrifugation (3000 rpm, 20 min at 4°C), and the ammonium sulfate precipitate formed between 0 and 50% saturation was collected by centrifugation (8000 rpm, 20 min at 4°C). The precipitate was dissolved in saline and dialyzed against 0.02 M phosphate buffer, pH 6.8. The dialysate was used as an anti-porcine pancreatic kallikrein A serum. The titer was 6 μg/ml of antigen solution (both porcine pancreatic kallikreins A and B) when determined by counter-current immunoelectrophoresis.

Double Immunodiffusion——The method of Ouchterlony was employed.[10] Agarose solution (1.0% in saline containing 0.02% NaN_3) was poured onto a glass plate (2.6×7.5 cm) to provide a 1 mm thick gel layer. Antigens and antibody (each, 5 μl) were separately added to the wells (diameter; 2 mm) and kept overnight at 4°C in a humid chamber. Thereafter the precipitin lines formed were observed directly.

Immunoelectrophoresis——This was carried out on 1.5% (w/v) agarose gel in 0.05 M veronal buffer, pH 8.6, for 2 h at 4°C with a cooling system at a constant current of 3 mA/cm as described by Scheidegger.[11] The development with antibody was carried out overnight at 4°C.

Results

Purification of Prokallikrein A

All of the following procedures were carried out at 0—2°C. Porcine pancreas (1.5 kg) was minced with a meat grinder and water extraction of prokallikrein was carried out in the presence of SBTI, benzamidine, ethylenediamine tetraacetic acid (EDTA) and *N*-ethylmaleimide as mentioned in our previous paper.[1] The water extract was centrifuged for 30 min at 6000 rpm and the supernatant was obtained. Solid ammonium sulfate was added to this solution and the precipitate formed between 30 and 75% saturation was collected. The precipitate was dissolved in H_2O containing antipain, chymostatin, leupeptin, pepstatin (final concentrations, each 2 μg/ml) and 10 mg of SBTI, and dialyzed for 12 h against H_2O. After centrifugation of the dialysate (6000 rpm, 30 min), the pH and electric conductivity of the supernatant were adjusted to 6.0 and 3.0 mmho/cm, respectively, and the solution was applied to a CM-cellulose column (5.0×80 cm) equilibrated with 3.0 mmho/cm ammonium acetate buffer, pH 6.0. Non-adsorbed substances were than applied to a DEAE-cellulose column and the substances adsorbed on the column were eluted (Fig. 1). As shown in Fig. 1, prokallikrein (fractions No. 76—126) was eluted before the active kallikrein (fractions No. 128—200) and they were almost completely separated. The prokallikrein fractions of DEAE–cellulose chromatography were pooled and applied to a DEAE-Sepharose CL-6B column. Then, the adsorbed substances were eluted with a linear gradient of ammonium acetate. As shown in Fig. 2, two distinct prokallikrein peaks were observed (a small amount of active kallikrein was detected in fractions overlapping with prokallikrein). Fractions No. 82—106 and 107—124 were separately pooled. It is well known that kallikrein obtained from autolyzed porcine pancreas has two main heterogenous components, *i.e.*, kallikreins A and B, which are usually separated by anion-exchange chromatographies.[9,12] Thus, the two components of prokallikrein shown in

Fig. 2 were called prokallikreins A and B in this paper in accord with the nomenclature of kallikrein obtained from autolyzed pancreas. Prokallikrein A fractions were further purified by preparative disc gel electrophoresis (Fig. 3). After this, 14.2% of active kallikrein was detected in the fractions of prokallikrein. This active kallikrein is presumably kallikrein spontaneously generated from prokallikrein during or after preparative disc gel electrophoresis, because we had pooled prokallikrein A fractions which contained little active kallikrein from

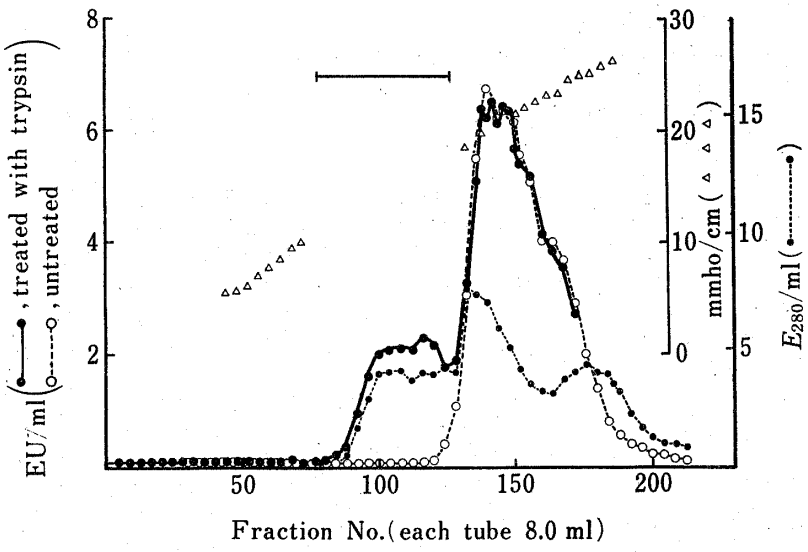

Fig. 1. DEAE-cellulose Chromatography of the Non-adsorbed Fractions from a CM-Cellulose Column

Fractions not adsorbed on a CM-cellulose column were directly applied to a DEAE-cellulose column (3.2×64 cm) equilibrated with 4.4 mmho/cm ammonium acetate, pH 6.3. Then, linear gradient elution with ammonium acetate, pH 6.0 (4.0 to 40 mmho/cm, total 2000 ml) was carried out. ⊢—⊣ in the figure shows the pooled fractions; the same symbol is used in the following figures (Figs. 2—5).

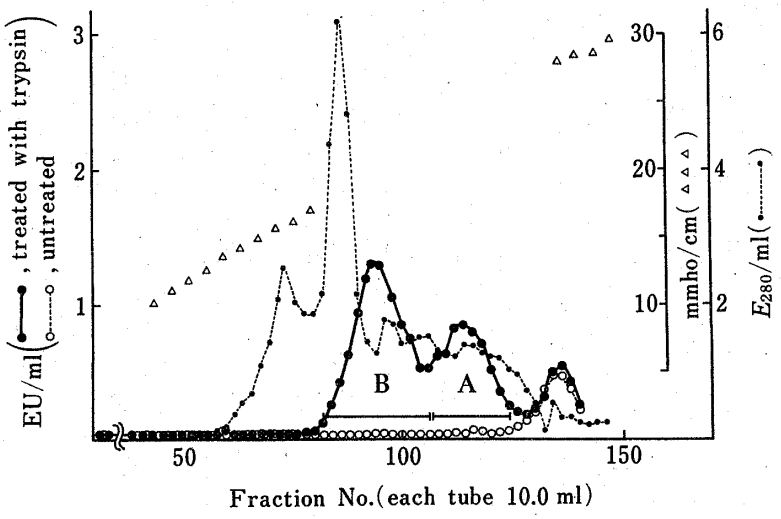

Fig. 2. Elution Profile of Prokallikrein from a DEAE-Sepharose CL-6B Column

Prokallikrein fractions in Fig. 1 were pooled and the electric conductivity of the solution was adjusted to 5.0 mmho/cm by addition of H_2O. Then, it was applied to a DEAE-Sepharose CL-6B column (2.0×62 cm) equilibrated with 5.0 mmho/cm ammonium acetate, pH 6.0, and linear gradient elution with ammonium acetate, pH 6.0 (5.0 to 35 mmho/cm, total 2000 ml) was carried out.

the previous DEAE-Sepharose CL-6B chromatography (Fig. 2). At present, the reason for this slight spontaneous activation of prokallikrein is not clearly understood. The same phenomenon (slight spontaneous activation during various chromatographies even under careful protection with various protease inhibitors) was observed in our previous investigation.[1,2] Fractions No. 50—63 were pooled and the 2nd DEAE-Sepharose CL-6B chromatography was carried out (Fig. 4). Active kallikrein observed in fractions No. 73—80 is presumably derived from the active kallikrein shown in the previous chromatogram (Fig. 3) which overlapped with prokallikrein, *i.e.*, active kallikrein spontaneously generated during or after the preparative disc gel electrophoresis was separated by the subsequent DEAE-Sepharose chromatography. Prokallikrein fractions (fractions No. 42—53 in Fig. 4) which also contained small amounts of active kallikrein were pooled and further gel-filtered on a Sephacryl S-200 column (Fig. 5). The prokallikrein fractions in Fig. 5 were divided into 3 portions and separately pooled, *i.e.*, (I): fractions No. 46—49, (II): fractions No. 42—45, (III): fractions No. 50—53 (see Fig. 5). Then, they were separately dialyzed against H_2O and the amount of prokallikrein and E_{280}/ml of the dialysates were determined. The esterase activities of the dialysates, I, II and III, determined after treatment with trypsin were 5.01, 1.24 and 1.11, respectively, and the E_{280}/ml values were 0.170, 0.063 and 0.136, respectively. Thus, the specific activities of I, II and III were calculated to be 29.5, 19.6 and

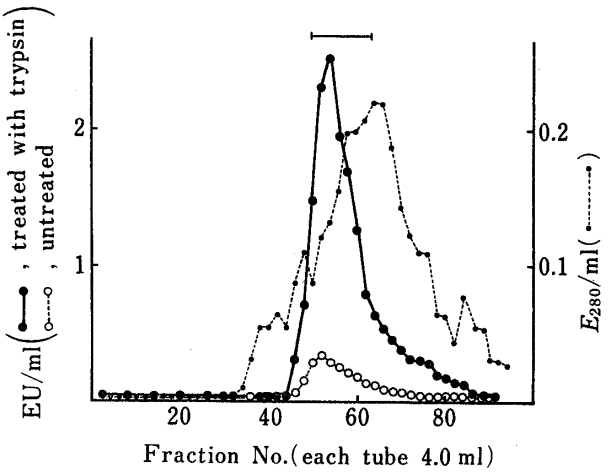

Fig. 3. Preparative Disc Gel Electrophoresis of Prokallikrein A obtained from DEAE-Sepharose CL-6B Chromatography

Prokallikrein A fractions (fractions No. 107—124 in Fig. 2) were pooled and dialyzed against H_2O for 10 h. The dialysate was concentrated to 10 ml and preparative disc gel electrophoresis was carried out on 10% (w/v) polyacrylamide gel at a constant current of 5 mA. Separating gel length; 6.0 cm.

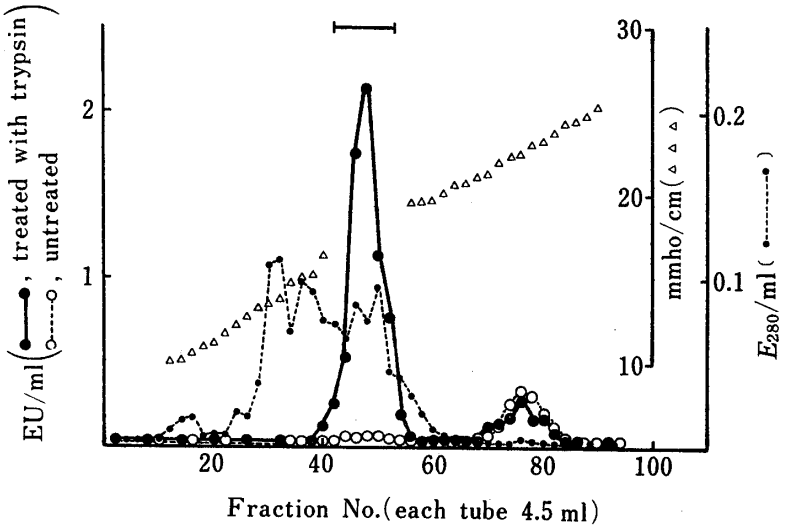

Fig. 4. Second DEAE-Sepharose CL-6B Chromatography of Prokallikrein A

Prokallikrein fractions obtained after the preparative disc gel electrophoresis were pooled, and the electric conductivity and the pH of the solution were adjusted to 4 mmho/cm and 6.0, respectively. The solution was applied on a DEAE-Sepharose CL-6B column (1.5×25.5 cm) equilibrated with 4.0 mmho/cm ammonium acetate, pH 6.0, and the adsorbed substances were eluted with a linear gradient with ammonium acetate, pH 6.0 (12 to 35 mmho/cm, total 500 ml).

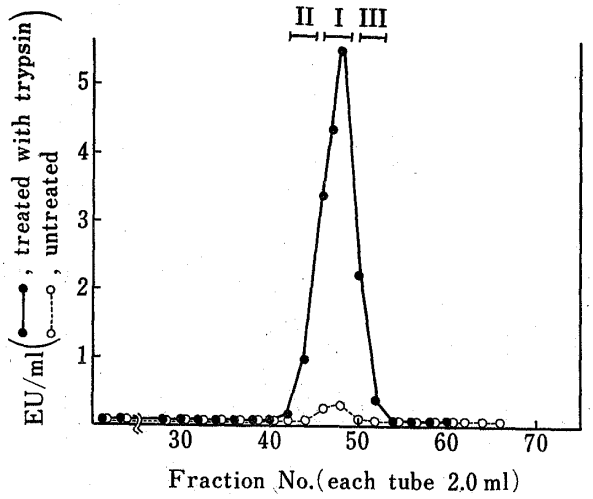

Fig. 5. Sephacryl S-200 Gel Filtration of Prokallikrein A obtained from the 2nd DEAE-Sepharose CL-6B Chromatography

Prokallikrein A fractions in Fig. 4 were pooled, concentrated to 2 ml and applied to a Sephacryl S-200 column (1.1×106 cm) equilibrated with 8 mmho/cm ammonium acetate, pH 6.0, containing 0.1 M NaCl.

8.2, respectively. The prokallikrein preparation (I), which had the highest specific activity, showed two distinct bands and two almost imperceptible bands when analyzed by disc gel electrophoresis on polyacrylamide gel (10%, w/v). Thus, complete purification has not yet been achieved, but this preparation, I in Fig. 5, was used in the following investigations as prokallikrein A preparation.

Table I summarizes the purification of prokallikrein A from the DEAE-cellulose chromatography to the last Sephacryl S-200 gel filtration (the esterase activities and the amounts of protein (absorbance at 280 nm) of the solutions obtained prior to the DEAE-cellulose chromatography could not be accurately determined because insoluble materials, such as fat, *etc.*, were emulsified in these solutions). As shown in this table, the prokallikrein preparation obtained after each chromatography contained a small amount of active form of kallikrein which appeared to have been spontaneously generated from prokallikrein during or after the chromatographies despite protection by various microbial protease inhibitors. We could not completely block this spontaneous activation, but the addition of these inhibitors to the elution buffers seemed to be very effective for the protection of prokallikrein; if these inhibitors were not used, extremely high contents of active form were frequently observed.

Table I. Summary of the Partial Purification of Porcine Pancreatic Prokallikrein A

Chromatographies[a]	Protein (E_{280})	Recovery (%)	Prokallikrein (EU)[b]	Recovery (%)	Specific activity (EU/E_{280})	P.F.
DEAE-cellulose	1367.7	100	572.5(4.1%)[c]	100	0.42	1
1st DEAE-Sepharose CL-6B	228.9	16.7	110.0(8.9%)	19.2	0.48	1.1
Preparative disc gel electrophoresis	9.9	0.7	101.0(14.2%)	17.6	10.2	24.3
2nd DEAE-Sepharose CL-6B	4.0	0.3	67.7(4.1%)	11.8	16.9	40.2
Sephacryl S-200[d]	1.78	0.12	52.6(6.3%)	8.8	29.5	69.5

a) Antipain, chymostatin, leupeptin and pepstatin were added to the elution buffer used (final concentrations, each 2 μg/ml).
b) Assayed after treatment with trypsin.
c) Contents of the active form of kallikrein (EU, untreated/EU, treated with trypsin ×100).
d) I in Fig. 5, *i.e.*, the main fractions in Fig. 5.

Immunoreactivity of Porcine Pancreatic Prokallikrein A

Figure 6 shows the double immunodiffusion analysis of porcine pancreatic prokallikrein A. Our final preparation reacted with the antibody against kallikrein A obtained from autolyzed porcine pancreas (this antibody reacts with both kallikreins A and B) and formed one precipitin line fused with that of the mixture of kallikreins A and B.

Figure 7 shows the immunoelectrophoretic patterns of prokallikrein A and the mixture

of kallikreins A and B. Prokallikrein A gave one precipitin line, as in the double immunodiffusion analysis (Fig. 6) but it migrated slightly more slowly than kallikreins A and B.

Molecular Weight of Prokallikrein A

The approximate molecular weight of prokallikrein A determined by gel permeation chromatography on interconnected Shodex A-803 and A-804 columns was $3.6—3.9\times10^4$ from the calibration curve shown in Fig. 8. On the other hand, the molecular weights of kallikreins A and B were estimated to be $3.3—3.4\times10^4$ on the same column. The values show the range of molecular weights obtained from 2 experiments.

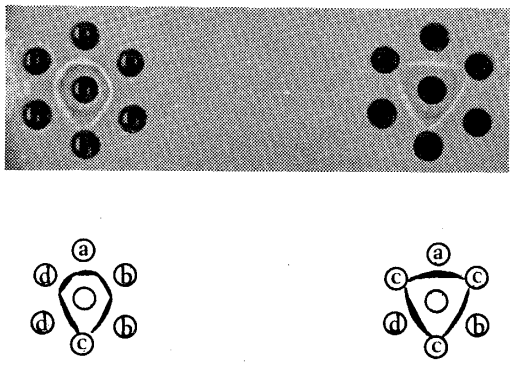

Fig. 6. Double Immunodiffusion Analysis of Porcine Pancreatic Prokallikrein A

Center well: antiserum against porcine pancreatic kallikrein A.
a : porcine pancreatic prokallikrein A solution (5.6 EU/ml).
b : mixture of porcine pancreatic kallikreins A and B solution (0.125 mg/ml).
c : saline.
d : mixture of porcine pancreatic kallikreins A and B solution (0.063 mg/ml).

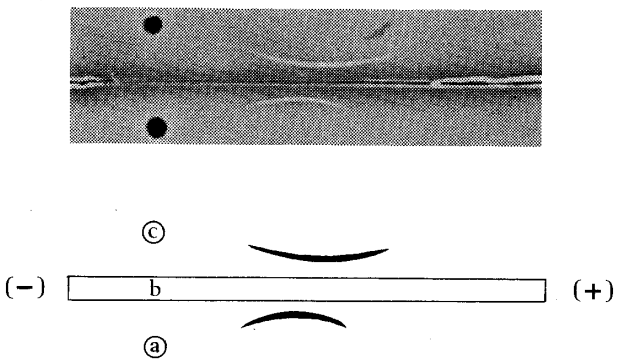

Fig. 7. Immunoelectrophorograms of Prokallikrein A and the Mixture of Kallikreins A and B

a : porcine pancreatic prokallikrein A solution (11.2 EU/ml).
b : antiserum against porcine pancreatic kallikrein A.
c : mixture of porcine pancreatic kallikreins A and B solution (1 mg/ml).

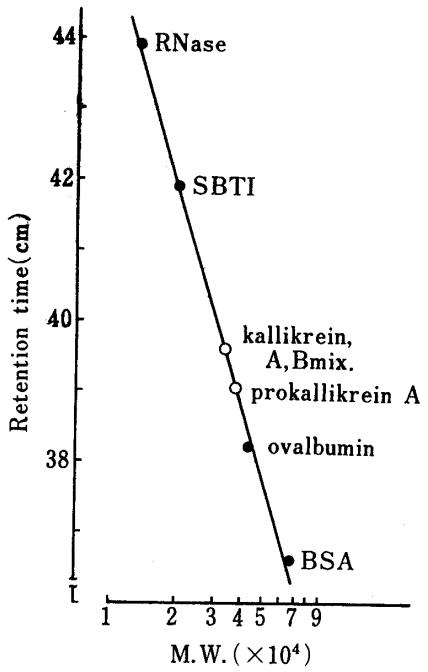

Fig. 8. Estimation of Molecular Weight of Porcine Pancreatic Prokallikrein A by Gel Permeation Chromatography on Interconnected Shodex A-803 and A-804 Columns

Authentic proteins used as standards were ribonuclease (M.W., 1.37×10^4), SBTI (M.W., 2.01×10^4), ovalbumin (M.W., 4.30×10^4) and bovine serum albumin (M.W., 6.7×10^4). The standard protein and the prokallikrein A solutions (100 μl each) were separately applied to a coupled combination of two Shodex A-803 columns and one Shodex A-804 column equilibrated with 0.1 M Tris–HCl buffer, pH 8.0, containing 0.1 M NaCl. The retention times (elution volumes) of the samples were automatically recorded on an autorecorder by measuring the absorbance at 280 nm. The retention time of prokallikrein A was confirmed by measuring the esterase activity of the eluates in addition to the measurement of the absorbance at 289 nm.

Discussion

It is well known that porcine pancreatic kallikrein obtained from autolyzed pancreas is usually separated into two main components, *i.e.*, kallikreins A and B, by anion-exchange chromatographies,[9,12] although the presence of a small amount of a third component separated

by the same chromatographies, such as kallikrein C, had been reported by some investigators.[13-15] On the other hand, the presence of two prokallikrein components, which can be separated by anion-exchange chromatography, has been reported by Fiedler *et al.*, and one of these two components, prokallikrein B, has been partially purified by them.[3a] The authors have also recognized the presence of two heterogenous prokallikrein components.[1,2] In the present work, we attempted to isolate one of these two prokallikrein components, prokallikrein A. The prokallikrein A preparation obtained here was stable as the pro-form; no detectable spontaneous activation was observed even when it was incubated for 1 h at 25°C. Our final prokallikrein A preparation had a specific activity of 29.5 EU/E_{280} (when assayed after activation with trypsin). Fiedler *et al.* reported that the specific activity of satisfactorily purified kallikrein B′, which is the active kallikrein spontaneously generated from prokallikrein B during its purification processes, was 72 EU/E_{280}.[3b] Judging from these values, the purity of our final prokallikrein A preparation was considered to be fairly high, although complete purification could not be achieved in the present work.

Our prokallikrein A immunologically reacted with the antibody against kallikrein A obtained from autolyzed porcine pancreas and formed one precipitin line fused with that of the mixture of kallikreins A and B (Fig. 6). Thus, it seems clear that our preparation is the zymogen of porcine pancreatic kallikrein. This view was also supported by the fact that the prokallikrein preparation in our previous paper,[1] which was the mixture of prokallikreins A and B, had potent vasodilator activity, one of the typical biological activities of kallikrein, when it was activated by trypsin.

On the other hand, the electrophoretic mobility of prokallikrein A was slightly slower than those of kallikreins A and B (Fig. 7). Thus, the isoelectric point of prokallikrein A was considered to be more basic than those of kallikreins A and B. This conclusion is consistent with the elution profile of prokallikrein A on DEAE-cellulose chromatography, *i.e.*, prokallikrein A was eluted at lower electric conductivity than active kallikrein (Fig. 1).

The molecular weight of prokallikrein A was estimated to be $3.6—3.9\times10^4$ by gel permeation chromatography, while those of kallikreins A and B were found to be $3.3—3.4\times10^4$ by the same method. Thus, it was speculated that at least large fragment would not be liberated from the prokallikrein A molecule during its activation by protease(s), although the most important enzyme(s) involved in the activation of prokallikrein and the detailed mechanism of activation of prokallikrein still remain to be elucidated.

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