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Porcine Pancreatic Prokallikrein. III. Some Different Forms of Kallikrein Generated from Prokallikrein[1)]

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Porcine pancreatic kallikreins generated from their precursors "prokallikreins A and B" in three different ways, *i.e.*, (1) kallikreins A and B obtained from autolyzed pancreas, (2) kallikreins A′ and B′ spontaneously generated during the purification of prokallikreins A and B and (3) kallikreins A″ and B″ generated from purified prokallikreins A and B by treatment with trypsin, were each highly purified and their properties were compared.

The K_m values for BzArgOEt hydrolysis, the mobilities on immunoelectrophoresis and the elution profiles from a DEAE-Sepharose CL-6B column of kallikreins A′ and B′ and kallikreins A″ and B″ closely resembled each other but apparently differed from those of kallikreins A and B. Kallikreins A and B were confirmed to consist of two polypeptide chains (three chains to some extent), while kallikreins A″, B″ and B′ were each determined to consist of a single chain. The amino acid compositions of the two-chain kallikrein and the single-chain kallikrein were very similar, though somewhat higher values of Leu, Glu and Lys residues were observed in the single-chain kallikrein as compared with the two-chain kallikrein.

Thus, kallikreins A″ and B″ generated from prokallikreins A and B by the action of trypsin were considered to be very similar to, or identical with, kallikreins A′ and B′ spontaneously generated during the purification of prokallikreins A and B. It is speculated that two-chain kallikreins A and B are generated from single-chain kallikreins A″ and B″ by the action of some protease(s) other than trypsin during the autolysis process.

Keywords——kallikrein; kallikrein–kinin system; prokallikrein; porcine pancreatic kallikrein; glandular kallikrein; activation; immunoelectrophoresis; amino acid composition

Kallikrein in porcine pancreas is considered to exist mainly as its inactive form "prokallikrein" and to be activated to its functional form "kallikrein" by the action(s) of protease(s) in the pancreas. However, several different forms of kallikrein generated from prokallikrein have been reported.[2)] According to that study,[2)] kallikreins A and B obtained from autolyzed porcine pancreas have two polypeptide chains (β-kallikrein). The presence of 10—20% of a three-polypeptide-chain kallikrein (γ-kallikrein) in the purified preparation of kallikrein B has also been reported.[3,4)] On the other hand, Fiedler *et al.* reported that spontaneous activation of prokallikrein B occurred during the purification procedures of prokallikrein B, especially in the gel filtration step, and almost all prokallikrein B was converted to active kallikrein.[5)] This spontaneously generated kallikrein, which was named kallikrein B′, has been shown to be a single-polypeptide chain kallikrein (α-kallikrein).

As described in our previous papers, we have succeeded in the partial purification of prokallikrein from porcine pancreas as a stable pro-form preparation and further succeeded in separating prokallikreins A and B.[1,6,7)] These prokallikrein preparations were rapidly activated by a trace amount of trypsin, which was the only factor that could significantly activate prokallikrein at a physiological concentration, though whether or not trypsin is the major activator of prokallikrein in normal physiological and/or pathological states in the

body is still unclear as yet. Thus, in order to clarify the true functional form of kallikrein in the living body and the enzyme(s) involved in the activation of prokallikrein in the body, we attempted to compare kallikreins A'' and B'' generated from isolated porcine pancreatic prokallikreins A and B by the action of trypsin with the other kallikreins mentioned above.

Materials and Methods

Materials——Fresh porcine pancreas was obtained from a slaughterhouse and stored at −25 °C until required. Trypsin from porcine pancreas (Type IX) and trypsin inhibitor from soybean (Type I-S) (SBTI) from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) were used for the assay of prokallikrein. Other reagents used were the same as mentioned in our previous papers.[1,6]

Esterolytic Activity Assay[8]——Esterolytic activity towards N^{α}-benzoyl-L-arginine ethyl ester (BzArgOEt) was measured photometrically as a kallikrein sasay, 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.[6]

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.[9] The development with antibody was carried out overnight at 4 °C.

SDS-Polyacrylamide Gel Electrophoresis——This was carried out as described by Laemmli.[10] Samples were treated with or without 5% (v/v) 2-mercaptoethanol in 0.0625 M Tris-HCl buffer, pH 6.8, containing both 2% (w/v) sodium dodecyl sulfate (SDS) and 20% (w/v) sucrose for 3 min at 100 °C. After being fixed with 12.5% (w/v) trichloroacetic acid, proteins were stained with 0.25% (w/v) Coomassie brilliant blue R-250 dissolved in MeOH–AcOH–H_2O (9:2:9, v/v).

Enzyme Purification——a) Prokallikreins A and B: Prokallikreins A and B were partially purified by our previously reported methods[1,6] and further by hydroxylapatite column chromatography (unpublished). The specific activities of the final preparations of prokallikreins A and B obtained here and used in the following experiments were 45.1 and 25.1 EU/E_{280}, respectively. b) Kallikreins A and B: Kallikreins A (103.7 EU/E_{280}) and B (107.8 EU/E_{280}) were purified from autolyzed porcine pancreas.[11] c) Kallikreins A* and B*: In order to block the activation of prokallikreins by the action(s) of protease(s) in the pancreas, we added various protease inhibitors to the extraction medium for the purification of prokallikreins from porcine pancreas.[1,6] However, considerable amounts of kallikreins were obtained as an active form which could be completely separated from prokallikreins by the first DEAE-cellulose chromatography as shown in Fig. 1 in ref. 6. These kallikreins generated even in the presence of various protease inhibitors (named kallikreins A* and B*) were purified according to almost the same procedures as used in the case of kallikreins A and B[11] and further by affinity chromatography on a column of aprotinin immobilized on Sepharose 4B (unpublished). The specific activities of the final preparations of kallikreins A* and B* were both 105.1 EU/E_{280}. d) Kallikreins A'' and B'': Prokallikreins A and B were separated by chromatography on a DEAE-Sepharose CL-6B column (see Fig. 2 in ref. 1) and the prokallikreins A and B fractions were separately pooled. Then, trypsin was added (final trypsin concentration, 2 μg/ml) and the solutions were allowed to stand for 6 h at room temperature then for a further 10 h at 4 °C to activate prokallikreins (kallikreins generated in this step were named kallikreins A'' and B''). The solutions were separately applied to a DEAE-Sepharose CL-6B column and the adsorbed materials were eluted. Figure 1 shows the case of kallikrein B''. Fractions No. 62—78 were pooled and further gel-filtered on a Sephacryl S-200 column (Fig. 2). Fractions No. 54—61 were pooled as a final kallikrein B'' fraction (105.1 EU/E_{280}). Kallikrein A'' was also purified (90.8 EU/E_{280}) by the same procedures as carried out in the case of kallikrein B''. e) Kallikreins A' and B': Kallikrein B' spontaneously generated from prokallikrein B during the purification of prokallikrein B[1,6] was further purified by means of the same chromatographies as carried out in the case of kallikreins A'' and B'', *i.e.*, DEAE-Sepharose CL-6B chromatography and gel filtration on a Sephacryl S-200 column. The specific activity of the final preparation of kallikrein B' was 87 EU/E_{280}. A sufficient amount of kallikrein A' could not be obtained here, so the kallikrein A' fractions shown in Fig. 4 in ref. 1, which were completely separated from prokallikrein A fractions, were used as kallikrein A' preparation.

Preparation of Anti-porcine Pancreatic Kallikrein Serum——The purified kallikreins A and B preparations were mixed in equal quantities and this mixture was used for the preparation of anti-porcine pancreatic kallikrein serum. The kallikrein solution (2.0 mg/ml), 1.0 ml, was emulsified with an equal volume of complete Freund's adjuvant and 1 ml of this emulsion (1 mg kallikrein eq) was injected intracutaneously into the footpads of rabbits (New Zealand white strain) weighing 2.5 kg. After 2 and 4 weeks, 1.5 ml of the same emulsion was injected intracutaneously into the footpads, and 6 weeks after the first injection the same volume of the emulsion was injected intracutaneously into the backs of the rabbits. The whole blood was collected one week after the last injection. The serum was obtained by centrifugation (1000 *g*, 10 min at 4 °C), and heated for 30 min at 56 °C. The titer was 128 when determined by the interfacial ring test using a solution of 1 mg/ml of antigen.

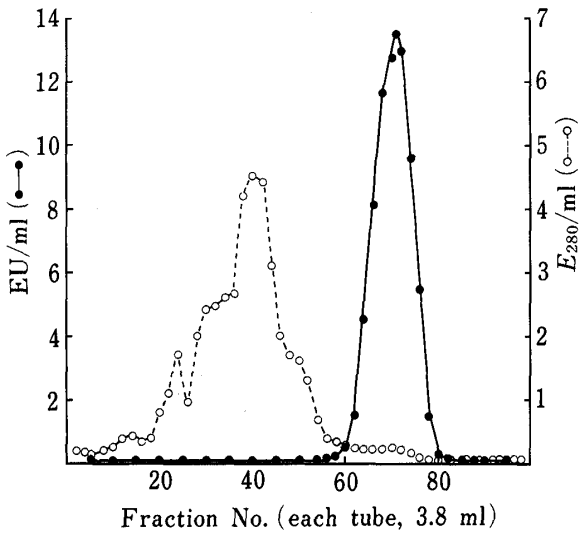


Fig. 1. Elution Profiles of Kallikrein B″ from a DEAE-Sepharose CL-6B Column

Prokallikrein solution obtained from the rechromatography on a DEAE-Sepharose CL-6B column was treated with trypsin as described in the text. The electric conductivity and the pH of this solution were adjusted to 5 mmho/cm and 6.0, respectively, and the solution was applied to a DEAE-Sepharose CL-6B column (1.5 × 25 cm) equilibrated with 5 mmho/cm of ammonium acetate, pH 6.0. The adsorbed materials were eluted with ammonium acetate, pH 6.0 (10 to 35 mmho/cm, total 500 ml).

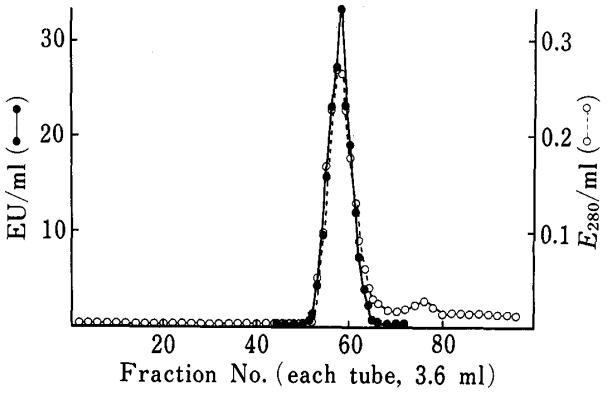


Fig. 2. Sephacryl S-200 Gel Filtration of Kallikrein B″ Obtained from DEAE-Sepharose CL-6B Chromatography

Kallikrein B″ fractions in Fig. 1 were pooled, concentrated to 8 ml and applied to a Sephacryl S-200 column (2.6 × 95 cm) equilibrated with 8 mmho/cm of ammonium acetate, pH 6.0. About 100 ml of the eluate before fraction No. 1 was not collected on the fraction collector.

Amino Acid Analysis——Amino acid analyses of 24, 48 and 72 h hydrolyzates of preparations of kallikrein B″ and B were performed on a Hitachi model 835 amino acid analyzer (Hitachi, Ltd., Tokyo, Japan). The molecular weight of kallikrein B″ has not been accurately determined as yet, so that the amino acid residues of kallikrein B″ were calculated on the basis of a molecular weight of 29000, the same as that of kallikrein B.[12]

Results

Immunoelectrophoresis

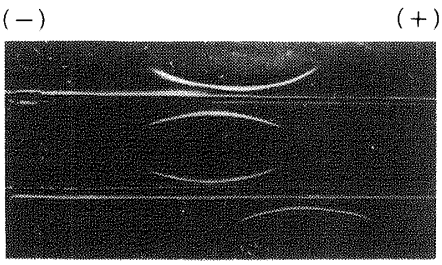
Figure 3 shows the result of immunoelectrophoresis of prokallikrein A, and kallikreins A and A″. The electrophoretic mobility of kallikrein A was the fastest and was apparently different from that of kallikrein A″. The mobility of pro-form kallikrein was the slowest among them.

Figure 4 shows the results for B form of kallikrein. The same relation of the electrophoretic mobilities among prokallikrein B, and kallikreins B and B″ was observed as in the case of the A form of kallikrein.

Elution Profiles from a DEAE-Sepharose CL-6B Column

Figure 5 summarizes the elution profiles of prokallikreins A and B, and various kallikreins from DEAE-Sepharose CL-6B columns of the same size. As shown in Fig. 5-I, prokallikrein A was eluted at the lowest ionic strength. Following prokallikrein A, kallikreins A′ and A″ were eluted at the same position and kallikrein A was eluted at the highest ionic strength.

The same elution order was observed in the kallikrein B group (Fig. 5-II). Thus, it appears that the molecules of kallikreins A and B obtained from autolyzed pancreas are different from those of kallikreins A′, A″, B′ and B″ generated from isolated prokallikreins A and B, and the molecules of kallikreins A″ and B″ generated by the action of trypsin were



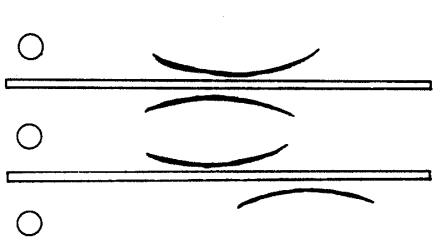
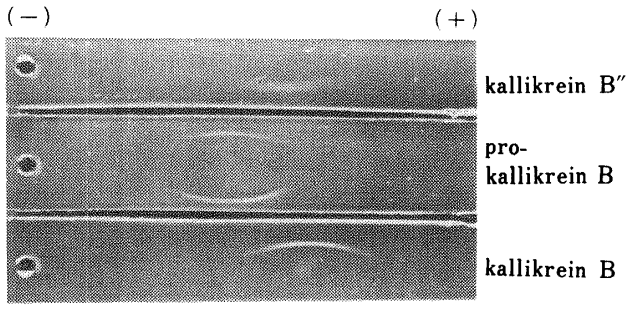


Fig. 3. Immunoelectrophorograms of Prokallikrein A, and Kallikreins A and A″

Seven μl aliquots of prokallikrein A (79.5 EU/ml), kallikrein A (98.4 EU/ml) and kallikrein A″ (77.6 EU/ml) solutions were separately added to the wells (diameter; 3 mm). After electrophoresis, 100 μl of antiserum against porcine pancreatic kallikreins A and B was added and developed overnight at 4 °C.



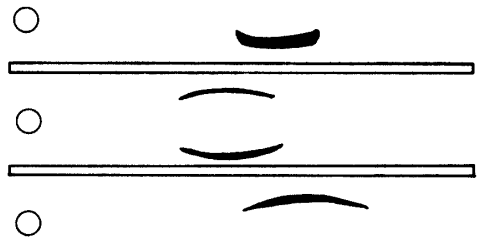


Fig. 4. Immunoelectrophorograms of Prokallikrein B, and Kallikreins B and B″

Solutions of prokallikrein B (19.0 EU/ml), kallikrein B (10.0 EU/ml) and kallikrein B″ (10.0 EU/ml) were used. The procedure was as described in the legend to Fig. 3.

considered to be identical or extremely similar to those of kallikreins A′ and B′ spontaneously generated during the purification of prokallikreins.

SDS-Polyacrylamide Gel Electrophoresis

Figure 6 shows the SDS-polyacrylamide gel electrophorograms of kallikreins A and A″ before and after reduction with 2-mercaptoethanol. Figure 6-I and -III show kallikreins A″ and A, respectively, before reduction with 2-mercaptoethanol. After reduction with 2-mercaptoethanol, the protein band of kallikrein A shown in Fig. 6-III disappeared and new bands were detected (Fig. 6-IV). The slower-migrating band in Fig. 6-IV was considered to correspond to the B-chain and the faster-migrating broad band was considered to be the A-chain of kallikrein A as judged from our previous work and other investigators' reports.[4,13] However, the presence of three neighboring bands could be recognized in this faster-migrating broad band. It was reported that γ-kallikrein is kallikrein whose B-chain has been further cleaved into two polypeptide chains which have similar molecular weight to the A-chain.[3,4] Thus, two of the three bands appearing in the faster-migrating band were considered to correspond to these chains derived from the B-chain. Thus, it is most likely that a considerable amount of γ-kallikrein was contaminating our present kallikrein A preparation. On the other hand, the protein band of kallikrein A″ (Fig. 6-I) was unchanged after reduction with 2-mercaptoethanol (Fig. 6-II), so this kallikrein was considered to consist of a single chain. A part of Table I summarizes the results of the analyses of polypeptide chains of other kallikreins on the same SDS-polyacrylamide gel electrophoresis. Namely, kallikreins A and B obtained from autolyzed pancreas were considered to consist of two or in part three chains and kallikreins A* and B* were also considered to consist of two chains. On the other hand, kallikreins A″ and B″ both consisted of a single chain. Kallikrein B′ was also a single-chain kallikrein.

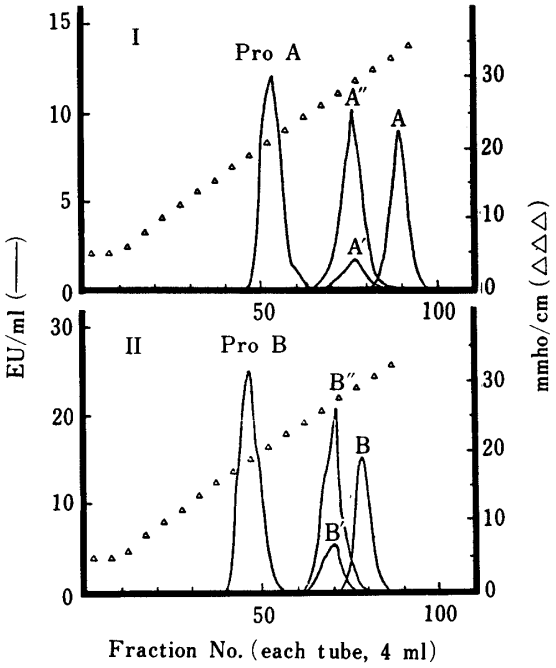


Fig. 5. Elution Profiles of Prokallikreins A and B, and Different Forms of Kallikrein Generated from Them, from a DEAE-Sepharose CL-6B Column

Arbitrary amounts of prokallikreins and kallikreins as shown in the figure, dissolved in 5 mmho/cm of ammonium acetate, pH 6.0, were separately applied to DEAE-Sepharose CL-6B columns (1.5 × 30 cm) equilibrated with the same buffer, and linear gradient elution with ammonium acetate, pH 6.0 (5 to 35 mmho/cm, total 400 ml) was carried out. In the case of kallikrein A, kallikrein was not completely eluted, so 35 mmho/cm ammonium acetate, pH 6.0, was further added to the column.

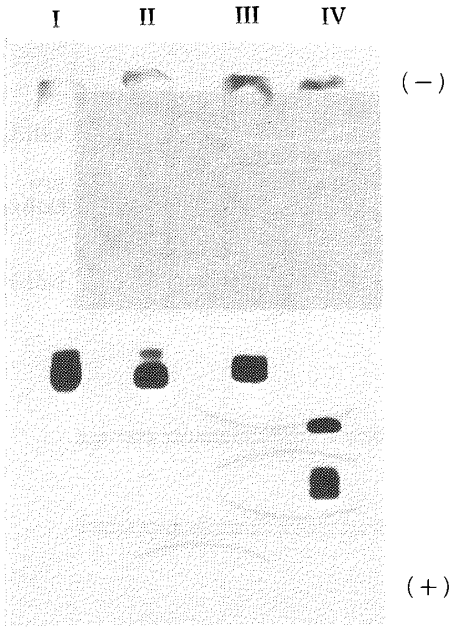


Fig. 6. SDS-Polyacrylamide Gel Electrophorograms of Kallikreins A and A'' before and after Reduction with 2-Mercaptoethanol

I: kallikrein A'', before the treatment with 2-mercaptoethanol.
II: kallikrein A'', after the treatment with 2-mercaptoethanol.
III: kallikrein A, before the treatment with 2-mercaptoethanol.
IV: kallikrein A, after the treatment with 2-mercaptoethanol.

K_m Values for BzArgOEt Hydrolysis

A part of Table I shows the K_m values of various kallikreins for BzArgOEt hydrolysis. The differences between single-chain kallikreins and two-chain kallikreins were not marked, but slightly higher values were observed in the single-chain kallikrein group. Furthermore, nearly equal K_m values were obtained for the kallikreins generated by the action of trypsin (kallikreins A'' and B'') and those spontaneously generated (kallikreins A' and B'). The same may be said for kallikreins A and B obtained from autolyzed pancreas and kallikreins A* and B* generated in the presence of various inhibitors.

Amino Acid Compositions of Kallikreins B'' and B

The amino acid compositions of kallikreins B'' and B were closely similar, though the contents of Glu, Leu and Lys in kallikrein B'' were somewhat higher than those of kallikrein B (two columns on the left in Table II). Whether the differences observed in these amino acids are due to experimental error is not clear as yet. The two columns on the right in Table II are the amino acid compositions of kallikreins B' and B reported by Fiedler *et al.*[5] and the center column is that of kallikrein B previously investigated in detail by us (Ikekita, unpublished).

Discussion

As mentioned in the introduction, several different forms of porcine pancreatic kallikrein

TABLE I. K_m Values of Various Forms of Porcine Pancreatic Kallikreins for BzArgOEt

Kallikreins	K_m (M)[a]
Kallikrein A (Two chains)[b]	1.3×10^{-4}
Kallikrein B (Two chains)[b]	1.2×10^{-4}
Kallikrein A* (Two chains)	1.6×10^{-4}
Kallikrein B* (Two chains)	1.3×10^{-4}
Kallikrein A′ (?)	2.0×10^{-4}
Kallikrein B′ (Single chain)	1.8×10^{-4}
Kallikrein A″ (Single chain)	2.1×10^{-4}
Kallikrein B″ (Single chain)	2.1×10^{-4}

a) pH 8.0 at 25 °C.
b) Preparations obtained here contained some γ-kallikrein.

TABLE II. Amino Acid Compositions of Porcine Pancreatic Kallikreins B″ and B

Amino acids	Kallikrein B″	Kallikrein B	Kallikrein B[a]	Kallikrein B′[5]	Kallikrein B[5]
Asp	27	27	29	27—28	28
Thr	14	14	16	15—16	15
Ser	13	13	13	14	14
Glu	27	23	24	23	23
Pro	16	17	17	16	16
Gly	19	20	24	21—22	22
Ala	14	13	14	13	13
Cys			5		10
Val	10	10	10	10	10
Met	3	2	2	4	4
Ile	12	12	12	11	12
Leu	28	22	22	21—22	20
Tyr	4	5	8	7	7
Phe	10	10	10	10	10
Lys	13	9	10	12	10
His	7	6	9	9	8
Arg	4	3	3	3	3
Trp			4		7
Total			232		232

a) Previously investigated in detail in our laboratory (unpublished).

have been reported. Figure 7 summarizes the possible relationships among kallikreins generated from prokallikreins A and B. Prokallikrein C is included because kallikrein C, a minor component of kallikrein obtained from autolyzed pancreas, has been reported by many laboratories and several components of prokallikrein other than the two main components were observed on isoelectric focusing fractionation of the crude prokallikrein preparation, as shown in our previous paper.[6] Therefore, kallikrein C′ and C″ may also be formed, though we could not confirm their presence as yet.

Fiedler *et al.* first reported that a spontaneous activation of prokallikrein occurred during gel filtration on Sephadex G-75, possibly due to the action of some unidentified contaminating

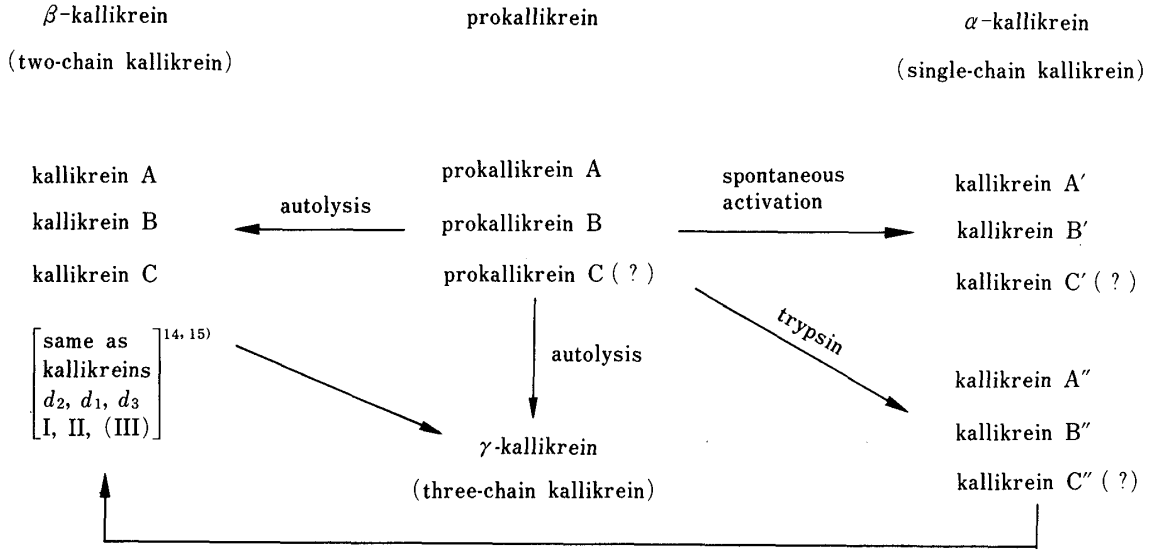


Fig. 7. Possible Relationship among Various Kallikreins Generated from Prokallikreins

protease, and this kallikrein was a single chain one.[5] In the present work, we confirmed their result, *i.e.*, this spontaneously generated kallikrein (kallikrein B′) consisted of a single chain. The chain strucrure of kallikrein A′ was not analyzed in the present work because we could not obtain a sufficient amount of kallikrein A′ preparation for analysis by SDS-polyacrylamide gel electrophoresis. However, kallikrein A′ may be expected to be a single chain. Kallikreins A″ and B″ generated from prokallikreins A and B by the action of trypsin were also shown to consist of a single chain, and the K_m values and the elution profiles from a DEAE-Sepharose CL-6B column of kallikreins A″ and B″ were almost the same as those of kallikreins A′ and B′ spontaneously generated from prokallikreins. In addition, the amino acid compositions of kallikreins B and B″ were closely similar, though somewhat higher values of Glu, Leu and Lys were observed in kallikrein B″. Fiedler *et al.* compared the amino acid compositions of kallikreins B and B′ and also observed that the contents of Leu and Lys residues of a single-chain kallikrein, kallikrein B′, were higher than those of kallikrein B (see Table II). Judging from these results, kallikreins A″ and B″ generated by the action of trypsin and kallikreins A′ and B′ spontaneously generated are concluded to be extremely similar or even identical.

Kallikreins A and B obtained from autolyzed pancreas consisted of two chains and in part three chains. Kallikreins A* and B* generated in the presence of various protease inhibitors in the water extraction step also consisted of two chains and their K_m values closely resembled those of kallikreins A and B obtained from autolyzed pancreas. Thus, these two groups of kallikreins were also considered to be closely similar or identical.

Trypsin is the only factor known to be able to activate prokallikrein significantly at a physiological concentration, and the present work showed that trypsin generates a single-chain kallikrein. Thus, we speculate that two-chain kallikreins A and B were generated from prokallikreins A and B by the action of both trypsin and some other protease(s) besides trypsin during the autolysis process. Therefore, single-chain kallikrein generated by the action of trypsin is considered to be the functional form in the body. However, it remains to be determined whether or not trypsin is the only ture activator of prokallikrein, because slight spontaneous activation during various chromatographies even in the presence of antipain and leupeptin, which are potent trypsin inhibitors, was always observed in our previous investigations.[1,6]

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