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Generation of α- and β-Kallikreins from Porcine Pancreatic Prokallikrein by the Action of Trypsin

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Prokallikrein was activated into α-kallikrein (single polypeptide chain) by the action of trypsin and further converted to β-kallikrein (two polypeptide chains) as indicated by the following observations;

1) When prokallikrein was activated with trypsin, rapid generation of kallikrein activity was observed followed by a slow decrease in the kallikrein activity during prolonged incubation. The same phenomenon of the slow decrease in the activity of α-kallikrein separately isolated was also observed on trypsin treatment.

2) From the results of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the kallikrein which was rapidly generated from prokallikrein by the action of trypsin was α-kallikrein. And the kallikrein obtained from α-kallikrein by further treatment with trypsin was β-kallikrein.

3) The N-terminal amino acid of α-kallikrein was isoleucine. In the case of β-kallikrein, two amino acids, isoleucine and alanine, were detected as N-terminal amino acids.

Conversion of α-kallikrein to β-kallikrein by the action of trypsin caused a decrease of N^{α}-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) hydrolyzing activity and changes of the kinetic constants for the hydrolysis of Bz-Arg-OEt. The kinetic constants of this β-kallikrein were distinctly different from those of the β-kallikrein obtained from autolyzed pancreas.

The above observation suggested that the β-kallikrein obtained in the present paper was a different type of β-kallikrein from that obtained from autolyzed pancreas.

Keywords——tissue kallikrein; porcine pancreatic kallikrein; prokallikrein; activation mechanism; trypsin; amino acid sequence; sodium dodecyl sulfate polyacrylamide gel electrophoresis; structure–activity relationship

Introduction

Porcine pancreatic kallikrein (one of the tissue kallikreins (EC 3.4.21.35)) is found in porcine pancreas as an inactive proenzyme, prokallikrein.[1] A few types of active kallikreins have been separately isolated from porcine pancreas and their properties have been investigated by several investigators.[2-4] Among them, two-chain β-kallikrein obtained from autolyzed pancreas (*i.e.* β-kallikreins A and B) has been initially characterized[3] and subjected to extensive experimental studies concerning the kallikrein–kinin system, as well as medical applications.

Previously, we succeeded in the purification of prokallikrein and demonstrated that prokallikrein (*i.e.* prokallikreins A and B) was activated by the action of low concentrations of trypsin, and the resulting active kallikrein was α-kallikrein (single chain, designated as

kallikreins A′′ and B′′).[2b] In the present work, we tried to elucidate the activation mechanism of prokallikrein by trypsin.

The present paper describes the activation mechanism of porcine pancreatic prokallikrein, *i.e.* the activation of prokallikrein to α-kallikrein, and the further formation of β-kallikrein from α-kallikrein by trypsin. The structure–activity relationships among α-kallikrein and β-kallikreins are also demonstrated.

Materials and Methods

Materials——Trypsin from porcine pancreas was purchased from Biozyme Lab. Ltd. (South Wales, Great Britain) and used after treatment with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK).[5] The esterolytic activity of this TPCK-treated trypsin was 29.0 EU/mg (21.2 EU/A_{280}). This TPCK-treated trypsin was used for prokallikrein activation. Soybean trypsin inhibitor (SBTI, type I-S) and *p*-nitrophenyl *p*′-guanidinobenzoate were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Bio-gel P-10 from Bio-Rad Lab. (Richmond, Ca., U.S.A.), molecular mass marker proteins from Pharmacia LKB Biotechnology (Uppsala, Sweden), nitrocellulose membrane (0.45 μm pore size, in roll form) from Millipore Co. (Bedford, Ma., U.S.A.), Vectastain ABC kit from Vector Lab. (Sunnyvale, Ca., U.S.A.) and 3,3′-diaminobenzidine tetrahydrochloride from Dojindo Lab. (Kumamoto, Japan) were also used. Carboxypeptidase P (CPase P) was purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Partially purified prokallikrein A preparation (20.1 EU eq/A_{280}) was obtained by Butyl-TOYOPEARL 650 M column chromatography (unpublished) from the prokallikrein A fraction which was obtained after the 1 st DEAE-Sepharose CL-6B anion-exchange chromatography in ref. 1*c*. Partially purified prokallikrein B (1.38 EU eq/mg) was obtained after the 1 st DEAE-Sepharose CL-6B anion-exchange chromatography in ref. 1*c*. Beta-kallikrein B (100 EU/A_{280}) was prepared as described previously.[2b] Anti-porcine pancreatic β-kallikrein B rabbit antiserum used was prepared in our laboratory according to the method previously described.[1c] Guanidinated aprotinin Sepharose 4B was prepared in our laboratory according to the method of Lemon *et al.*[6] Other reagents used were the same as mentioned in our previous papers.[1c,d,2b]

Preparation of Trypsin-Immobilized Sepharose 4B——The TPCK-treated trypsin mentioned above was further purified on an SBTI-Sepharose 4B column. The specific activity of the final trypsin preparation was 36.4 EU/A_{280}. This trypsin, 236.6 EU, was coupled on 13 ml of CNBr-activated Sepharose 4B gel according to the method of Stewart and Doherty.[7]

Esterolytic Activity Assay——Esterolytic activities of kallikrein and trypsin toward N^{α}-benzoyl-L-arginine ethyl ester (Bz–Arg–OEt) were determined photometrically according to the method of Schwert and Takenaka[8] with minor modifications. The final substrate concentration was 1 mM and one esterase unit (EU) was defined as the amount of enzyme that could hydrolyze 1 μmol of substrate per min at 25°C, pH 8.0.

The amount of prokallikrein was determined as newly generated activity[1d] and expressed as EU equivalent (EU eq).

Determination of Kinetic Constants for the Hydrolysis of Bz–Arg–OEt——The hydrolysis of Bz–Arg–OEt was followed photometrically at pH 8.0 and 25 °C, and the substrate concentrations used were as given in the footnote of Table II. To obtain the k_{cat} value, active enzyme concentration was determined by titration with *p*-nitrophenyl *p*′-guanidinobenzoate according to the method of Fiedler *et al.*[9]

Purification of Kallikrein B′′——Purification of kallikrein B′′ was performed as follows. Partially purified prokallikrein B powder, 503.9 mg, was dissolved in 114 ml of 50 mM triethanolamine hydrochloride–NaOH buffer, pH 7.0, containing 10 mM $CaCl_2$, and preincubated for 10 min at 37 °C. Then, 2 ml of trypsin solution (14.5 mg/ml in 0.01 N HCl) was added and the mixture was incubated at 37°C (final trypsin concentration, 250 μg/ml). After a 10 min incubation, 5 ml of SBTI solution (17.4 mg/ml in 50 mM triethanolamine hydrochloride–NaOH buffer, pH 7.0, containing 10 mM $CaCl_2$) was added in order to inactivate trypsin. The resulting active kallikrein, designated as kallikrein B′′ was further purified by DEAE-Sepharose CL-6B anion-exchange chromatography, guanidinated aprotinin Sepharose 4B affinity chromatography and Sephacryl S-200 gel filtration. A kallikrein B′′ preparation which had a specific activity of 82.3 EU/A_{280} (total 438.7 EU) was obtained in a yield of 63.8% from the step after activation of prokallikrein B by trypsin, and was stored at −20 °C after lyophilization.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)——This was performed as described by Laemmli (gel concentration: 12.5%).[10] After the electrophoresis, the gels were stained with Ag-stain DAIICHI kit (Daiichi Pure Chemical Co., Ltd., Tokyo, Japan) or Coomassie Brilliant Blue R-250.

Protein Blotting and Detection of Kallikrein——After SDS-PAGE, proteins were electrophoretically transferred to a nitrocellulose sheet in a Bio-Rad Trans-blot device according to the method of Towbin *et al.*[11] After transfer, the blot was treated with anti-β-kallikrein B rabbit antiserum followed by biotin-labeled anti-rabbit immunoglobulin G (IgG) and avidin–biotin–peroxidase complex (Vectastain ABC kit). Then the blot was developed in a peroxidase substrate solution (0.05% (w/v) 3,3′-diaminobenzidine tetrahydrochloride and 0.01% (v/v) H_2O_2 in 0.05 M Tris–HCl

buffer, pH 7.2) for about 10 min at room temperature.

Analyses of N-Terminal Amino Acid Sequence and C-Terminal Amino Acid——Automated amino acid sequence analyses of the N-terminal region of porcine pancreatic kallikreins were performed with a gas phase protein sequencer (model 477A Protein Sequencer, connected to a 120A PTH Analyzer, Applied Biosystems, Foster City, Ca., U.S.A.). The C-terminal amino acid of porcine pancreatic β-kallikrein was determined by the carboxypeptidase method using CPase P. Beta-kallikrein B (0.5 nmol) and Kallikrein B''-T (0.32 nmol) were dissolved separately in 50 μl of 0.1 M pyridine–acetic acid (pH 5.5) containing 1% (v/v) 2-mercaptoethanol. Then the enzyme solution was heated at 100 °C for 10 min. After cooling, 5 μl of CPase P solution (1 mg/ml in H_2O) was added and the mixture was incubated at 37 C for 1 h. The released amino acids were analyzed with an Irika amino acid analyzer, model A-5500 (Irika Instruments, Inc., Kyoto, Japan).

Determination of Amino Acid Sequence of the N-Terminal Region of Prokallikrein——Partially purified prokallikrein A (20.1 EU eq/A_{280}), 12.6 EU eq, was further purified by SDS-gel electrophoresis and prokallikrein A was extracted from the SDS-gel according to the method of Tsugita *et al.*[12] Then, the extracted prokallikrein A was dissolved in 50 μl of 0.1 M pyridine–acetate–collidine buffer containing 0.1% (w/v) SDS (pH 8.0) and heated at 100 °C for 5 min. After cooling, the mixture was evaporated. The residue was redissolved in 50 μl of 70% formic acid and applied to the protein sequencer.

Results and Discussion

Activation Profiles of Prokallikrein B by Trypsin

Figure 1A shows the relationships between the amount of trypsin used and generated esterolytic activity when the partially purified prokallikrein B preparation was incubated with various concentrations of trypsin solution for 10 min at 37 °C. The maximum activation was observed at the trypsin concentration of 250 μg/ml, and the activity decreased at trypsin concentrations of more than 500 μg/ml.

Figure 1B shows the effect of incubation time on the activation of prokallikrein B with trypsin at the final concentration of 333 μg/ml. At this concentration, prokallikrein B was rapidly activated by trypsin and the maximum kallikrein activity was obtained within 10 min. However, a slow decrease of the esterolytic activity was observed during prolonged incubation. These observations suggested that prokallikrein was rapidly activated by trypsin

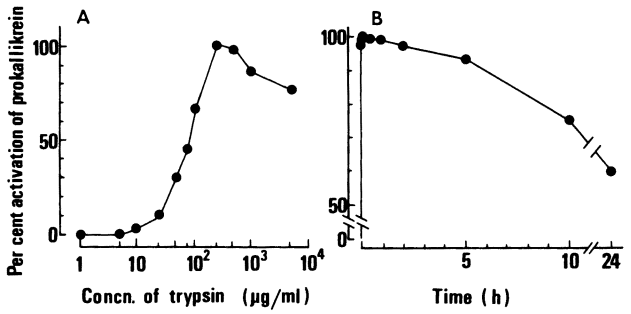

Fig. 1. Activation Profiles of Porcine Pancreatic Prokallikrein B by Trypsin

(A) Partially purified prokallikrein B (1.38 EU eq/mg) solution (0.2 ml) (6.9 EU eq/ml in 50 mM triethanolamine hydrochloride–NaOH buffer, pH 7.0, containing 10 mM $CaCl_2$) was preincubated for 5 min at 37 °C. Then, 0.1 ml of trypsin solution was added (final trypsin concentration, 1, 5, 10, 25, 50, 75, 100, 250, 500 μg, 1 mg or 5 mg/ml) and the mixture was incubated at 37 °C. After 10 min, 0.1 ml of SBTI solution (the concentration of SBTI solution used was three times as high as the indicated final trypsin concentration by weight) was added and the reaction mixture was allowed to stand for 10 min at room temperature. Then, the esterolytic activity of the mixture was measured. (B) Partially purified prokallikrein B (1.38 EU eq/mg) solution (6.9 EU eq/ml in 50 mM triethanolamine hydrochloride–NaOH buffer, pH 7.0, containing 10 mM $CaCl_2$, 0.2 ml each) was preincubated at 37 °C for 5 min. Trypsin solution (1 mg/ml in the same buffer), 0.1 ml, was added to each of the prokallikrein solutions and the mixture was incubated at 37 °C (final trypsin concentration, 333 μg/ml). After the indicated incubation periods, 0.1 ml of SBTI solution (3 mg/ml in the same buffer) was added and the mixture was left for 10 min at room temperature. Then the esterolytic activity of the mixture was measured. The amount of prokallikrein activated was expressed as per cent of maximum activity obtained.

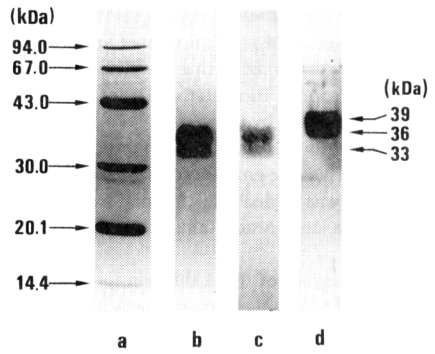

Fig. 2. SDS-PAGE Analyses of Kallikrein B″

Lane (a): Molecular mass markers (phosphorylase b, 94.0 kDa; albumin, 67.0 kDa; ovalbumin, 43.0 kDa; carbonic anhydrase, 30.0 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa).

Lane (b): Non-reduced kallikrein B″, 0.03 EU, was applied to the gel. The gel was stained with silver.

Lane (c): After the same electrophoresis as in (a), proteins were electrophoretically transferred to a nitrocellulose sheet and kallikrein B″ was immunologically detected.

Lane (d): Kallikrein B″, 0.03 EU, was reduced with 2-mercaptoethanol and applied to the gel. The gel was stained with silver.

TABLE I. Amino Acid Sequences of N-Terminal Regions of Prokallikrein A, Kallikrein B″ and Kallikrein B″-T

Cycle No.	Prokallikrein A		Kallikrein B″(α)		Kallikrein B″-T(β)	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Ala	752	Ile	945	Ala	756
					Ile	634
2	Pro	428	Ile	765	Ile	483
					Asp	216
3	Pro	371	Gly	1043	Gly	942[b]
4	Ile	527	Gly	1047	Gly	602
					Lys	243
5	Gln	227	Arg	288	Asp	274
					Arg	166
6	Ser	68	Glu	424	Tyr	398
					Glu	278
7	Arg	186	X[a]		Ser	95
8	Ile	357	Glu	370	Glu	252
					His	84
9	Ile	556				
10	Gly	283				
11	Gly	368				
12	Arg	233				

a) Not identified. *b*) As the yield of glycinyl residue was about 1.5 times the amount of kallikrein B″-T used, two glycinyl residues must have been liberated simultaneously from two N-terminal sites at this cycle. The amounts of intact kallikrein B″ and B″-T used for sequence analysis were 1 nmol and 0.65 nmol, respectively.

and the resulting kallikrein (designated as kallikrein B″) was further converted into another type of kallikrein (designated as kallikrein B″-T) which still had a low Bz–Arg–OEt hydrolyzing activity.

Analyses of Kallikrein B″ on SDS-PAGE and Amino Acid Sequence of the N-Terminal Region

Figure 2 shows the SDS-PAGE of kallikrein B″ purified from prokallikrein B. Doublet bands, whose molecular masses were estimated to be 36 kilo daltons (kDa) and 33 kDa, respectively, were observed (Fig. 2b), and identical bands were detected after immunological blotting using anti-β-kallikrein B antibody (Fig. 2c). On the other hand, under reducing conditions, kallikrein B″ appeared as one broad band apparently composed of two bands, corresponding to 39 and 36 kDa (Fig. 2d). A single N-terminal amino acid of kallikrein B″ was detected, isoleucine, and its amino acid sequence was determined as H–Ile–Ile–Gly–Gly–

Arg–Glu–( )–Glu- (Table I). From these observations it was concluded that kallikrein B′′ was a single-chain α-kallikrein and consisted of two molecular mass variants. It was speculated that the two molecular mass variants of kallikrein B′′had the same protein moiety and differed in their carbonhydrate moiety. Beta kallikrein obtained from autolyzed pancreas has been separated into two forms, A and B, on ion exchange chromatography, and β-kallikreins A and B only differ in their attached carbohydorate, having the same protein moiety.[3a,c]

The amino acid sequence of the N-terminal region of kallikrein B′′ was identical with that of the A chain of β-kallikrein B prepared from autolyzed pancreas, H–Ile–Ile–Gly–Gly–Arg–Glu–Cys–Glu–.[13]

Further Treatment of Kallikrein B′′ with Trypsin

Kallikrein B′′ was incubated with trypsin-immobilized Sepharose 4B at 37 °C, pH 7.0, for the indicated periods in Fig. 3 and the remaining esterolytic activity was measured. As shown in Fig. 3, the esterolytic activity gradually decreased with increasing incubation time and reached a plateau at 24 h (about 60% of the initial activity). This phenomenon was also analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 4, protein bands of kallikrein B′′ gradually disappeared with the passage of incubation time and three bands corresponding to 25.5, 17.5 and 14.4 kDa were newly observed. Thus, we designated the kallikrein derivatives as kallikrein B′′-T.

Polypeptide Chain Structure of Kallikrein B′′-T

The obtained kallikrein B′′-T was also found to consist of two molecular mass variants (36 and 33 kDA), like kallikrein B′′, and was different from β-kallikrein B from autolyzed pancreas (29 kDa) in SDS-PAGE under non-reducing conditions (Fig. 5). On the other hand,

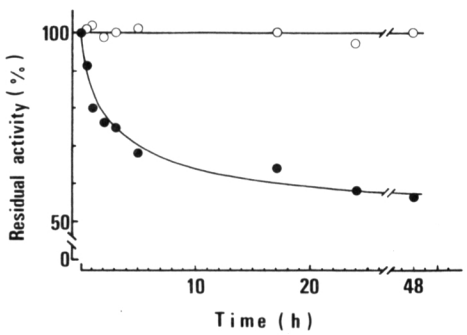

Fig. 3. Effect of Trypsin on the Esterolytic Activity of Kallikrein B′′

Purified kallikrein B′′ solution (62 EU in 5.25 ml of 50 mM triethanolamine hydrochloride–NaOH buffer, pH 7.0, containing 10 mM $CaCl_2$) was mixed with 5.5 ml of trypsin-immobilized Sepharose 4B which had been equilibrated with the same buffer and incubated at 37 °C with continuous stirring. After the indicated incubation period, an aliquot (1 ml) was taken from the incubation mixture followed by removal of the gels, then the esterolytic activity of the solution toward Bz–Arg–OEt was measured and SDS-PAGE analysis of the solution was performed (see Fig. 4). The esterolytic activity of kallikrein B′′ before treatment with trypsin-immobilized Sepharose 4B was taken as 100%. The symbols, (●) and (○) indicate treatment with and without trypsin-immobilized Sepharose 4B, respectively.

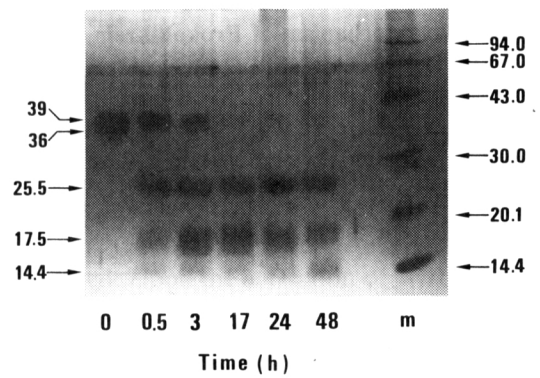

Fig. 4. Generation of β-Kallikrein from Kallikrein B′′ by the Action of Trypsin

After kallikrein B′′ had been treated with trypsin-immobilized Sepharose 4B gels at 37 °C, pH 7.0, for the indicated time, the gels were removed and the solution was obtained (see the legend to Fig. 3). Then 200 μl of the solution was dialyzed against water and the dialysate, 100 μl, was mixed with the same volume of 0.125 M Tris–HCl buffer, pH 6.8, containing 10% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 12% (v/v) glycerol and 0.005% (w/v) bromophenol blue. The mixture was placed in a boiling water bath for 5 min. Twenty μl of it was applied to the gel. The sample used at 0 time was prepared by using water instead of trypsin-immobilized Sepharose 4B gels. Lane (m): Molecular mass markers (see the legend to Fig. 2).

under reducing conditions this kallikrein B′′-T was found to consist of a heavy chain of 25.5 kDa, which coincided with the B chain of β-kallikrein B, and two light chains of 17.5 and 14.4 kDa which should be molecular mass variants (differing in the amount of attached carbohydrate) (Fig. 5).

Kallikrein B′′-T was found to have two N-terminal amino acids, isoleucine and alanine (Table I). In sequence analysis of the N-terminal region, two amino acid residues were detected at each cycle except for the 7th cycle. In addition, one of two amino acids in each cycle was identical with the N-terminal sequence of kallikrein B′′. Consequently, kallikrein B′′-T was revealed to have the following two N-terminal sequences, H–Ile–Ile–Gly–Gly–Arg–Glu–( )–Glu– and H–Ala–Asp–Gly–Lys–Asp–Tyr–Ser–His–. The former was identical with the amino acid sequence of the N-terminal region of kallikren B′′ and also with that of the A chain of β-kallikrein B, while the latter was identical with that of the B chain of β-kallikrein B molecule.[13]

C-Terminal amino acid analyses of β-kallikrein B (0.5 nmol) and kallikrein B′′-T (0.35 nmol) by the use of CPase P showed that only leucine (0.23 nmol) and lysine (0.31 nmol) were detected as the C-terminal amino acid of β-kallikrein B and kallikrein B′′-T, respectively. Leucine, found in the case of β-kallikrein B, was identical with the C-terminal amino acid of the A chain as previously reported.[14] We could not detect any proline, which was anticipated to be the C-terminal amino acid of the B chain. It was considered that proline was not released from the C-terminus of both the B chain of β-kallikrein B and heavy chain (= B chain) of kallikrein B′′-T by CPase P under the present conditions. Consequently, lysine detected in the case of kallikrein B′′-T was regarded as the C-terminal amino acid of the light chain.

Thus, kallikrein B′′-T consisted of two polypeptide chains, being a β-type kallikrein, but it was different in structure from β-kalikrein B itself (see Fig. 7).

Activation Mechanism of Prokallikrein by Trypsin

In the present study, the amino acid sequence of the N-terminal region of porcine pancreatic prokallikrein A was determined (Table I). Figure 6 shows a comparison of this amino acid sequence with those of prokallikreins previously reported.[15–18] The sequence of seven amino acids in the N-terminal region of our prokallikrein A, so-called propeptide, was

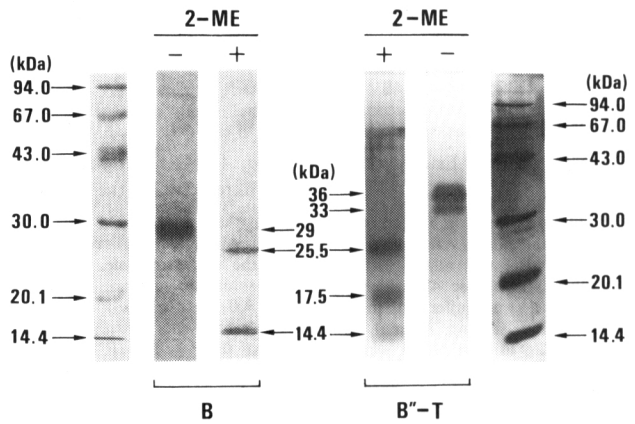

Fig. 5. SDS-PAGE Analyses of β-Kallikreins B and B′′-T

Each β-kallikrein B (10 μg) and β-kallikrein B′′-T (0.03 EU) was separately treated with (+) or without (−) 2-mercaptoethanol (2-ME) and applied to the gel. The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 and silver, respectively. Lane (m): molecular mass markers (see footnote in Fig. 2).

	−7				−1	+1				+5
porcine pancreatic prokallikrein[a)]	H–Ala–	Pro–	Pro–	Ile–	Gln–	Ser–	Arg–	Ile–	Ile–	Gly–Gly–Arg–
human pancreatic prokallikrein[15)]	–Ala–	Pro–	Pro–	Ile–	Gln–	Ser–	Arg–	Ile–	Val–	Gly–Gly–Trp–
human urinary prokallikrein[16)]	H–Ala–	Pro–	Pro–	Ile–	Gln–	Ser–	Arg–	Ile–	Val–	Gly–Gly–Trp–
rat pancreatic prokallikrein[17)]	–Ala–	Pro–	Pro–	Val–	Gln–	Ser–	Arg–	Val–	Val–	Gly–Gly–Tyr–
mouse submaxillary grand prokallikrein[18)]	–Ala–	Pro–	Pro–	Val–	Gln–	Ser–	Arg–	Ile–	Val–	Gly–Gly–Phe–

propeptide region

Fig. 6. Comparison of Prokallikreins

The amino acid sequence at positions −7 to −1 is the so-called propeptide region. *a*) This work. The amino acid sequences of human[15)] and rat[17)] pancreatic prokallikreins and mouse submaxillary grand prokallikrein[18)] are those predicted from the nucleotide sequences.

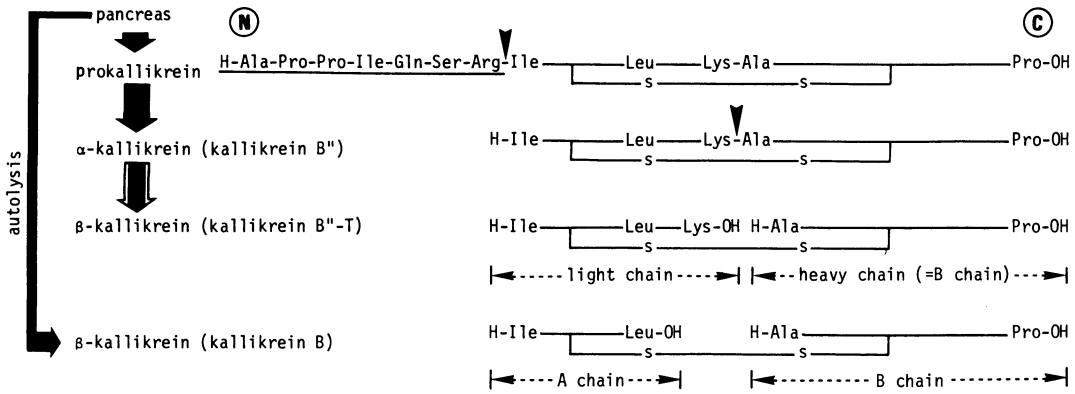

Fig. 7. Assigned Polypeptide Chain Structure Relationship among Porcine Pancreatic Prokallikrein and α- and β-Kallikreins

The underlined N-terminal portion of prokallikrein is the so-called propeptide region. Arrowheads indicate the sites of tryptic cleavage suggested in the present study. Symbols, Ⓝ and Ⓒ indicate N-terminal and C-terminal sites of porcine pancreatic prokallikrein and kallikreins, respectively.

identical with that of human prokallikreins,[15,16)] and was highly homologous to those of other prokallikreins.[17,18)]

In Fig. 7, the assigned polypeptide chain structures of our porcine pancreatic prokallikrein and the generated kallikreins are summarized. Upon the activation of prokallikrein to α-kallikrein (kallikrein B′), the Arg–Ile bond was the site of tryptic cleavage and the N-terminal peptide consisting of 7 amino acids was simultaneously released from the prokallikrein molecule (the rapidly activated state).

Furthermore, α-kallikrein (kallikrein B′) was converted into β-kallikrein (kallikrein B′-T) by further action of trypsin (the state of decreased activity). The Lys–Ala bond which linked light to heavy chain should be cleaved by trypsin in this state.

The missing portion between the A and B chains of β-kallikrein B coming from autolyzed pancreas has been designated as the 'kallikrein autolysis loop' and was still preserved in the α-kallikrein molecule, *i.e.* kallikrein B′.[14)] It was concluded that kallikrein B′-T retained the 'kallikrein autolysis loop' in the C-terminal region of the light chain. This clearly indicates the difference between kallikrein B′-T and β-kallikrein B, as previously mentioned.

The mechanism of the artificial autolysis process of prokallikrein is still obscure, but trypsin is considered to play an important part in the process. After generation of kallikrein B′-T from prokallikrein B by the action of trypsin, further conversion into β-kallikrein B should occur by removal of the 'kallikrein autolysis loop' by some protease (s) other than trypsin present in the pancreas.

TABLE II. Kinetic Constants for the Hydrolysis of Bz–Arg–OEt by Kallikreins B′′, B′′-T and B

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
α-Kallikrein B′′	0.234±0.007	62.5±1.2	267
β-Kallikrein B′′-T	0.333±0.023	53.8±2.5	162
β-Kallikrein B	0.109±0.005	81.6±2.3	749

Each K_m and k_{cat} value of α-kallikrein B′′, β-kallikrein B′′-T was estimated at Bz–Arg–OEt concentrations of 0.1—1.0 mM. The K_m and k_{cat} values of β-kallikrein B were estimated at Bz–Arg–OEt concentrations of 0.05—1.0 mM. Values of K_m and k_{cat} are means±S.E. for three determinations.

Kinetic Constants for the Hydrolysis of Bz–Arg–OEt by Kallikreins Generated from Prokallikrein

Kinetic constants for the hydrolysis of Bz–Arg–OEt by kallikreins B′′, B′′-T and B are shown in Table II. The k_{cat}/K_m value of β-kallikrein B was the highest among these three kallikreins. The k_{cat}/K_m value of kallikrein B′′-T was the lowest, being about 60% of that of kallikrein B′′. These results suggest that the cleavage of the Lys–Ala bond (generation of kallikrein B′′-T) and the removal of the 'kallikrein autolysis loop' (generation of β-kallikrein B) had a marked influence on the kinetic properties of kallikrein B′′.

From these observations, it was concluded that prokallikrein was activated into α-kallikrein, which was further converted into β-kallikrein by trypsin. A similar activation mechanism was recently reported for human urinary prokallikrein by Takahashi *et al.*[19] It is noteworthy in the present study that conversion of α- to β-kallikrein altered the kinetic properties toward a synthetic substrate, Bz–Arg–OEt. In nature, the substrate (s) should be large molecule proteins, so that the 'kallikrein autolysis loop' might have an interesting role in the regulation of kallikrein activity. In the case of the activation mechanism of human plasma kallikrein, proteolytic cleavage of the heavy chain of plasma α-kallikrein resulted in the formation of plasma β-kallikrein accompanied with a lowering of coagulant and kininogenase activities and loss of the ability to activate blood polymorphonuclear leukocytes.[20] It would be very interesting to investigate the process of activation of tissue prokallikrein *in vivo* by protease (s) to clarify the physiological and pathological roles of tissue kallikreins in the body.

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References

1) *a*) F. Fiedler and E. Werle, *Biol. Chem. Hoppe-Seyler*, **348**, 1087 (1967); *b*) F. Fiedler, C. Hirshauer and E. Werle, *ibid.*, **351**, 225 (1970); *c*) K. Kizuki, M. Kamada, M. Ikekita and H. Moriya, *Chem. Pharm. Bull.*, **30**, 3354 (1982); *d*) K. Kizuki, H. Murakami, M. Hiratsuka, M. Kamada and H. Moriya, *ibid.*, **34**, 4394 (1986).
2) *a*) F. Fiedler and W. Gebhard, *Biol. Chem. Hoppe-Seyler*, **361**, 1661 (1980); *b*) K. Kizuki, H. Takiguchi, M. Kamada, M. Ikekita and H. Moriya, *Chem. Pharm. Bull.*, **32**, 3662 (1984).
3) *a*) F. Fiedler, C. Hirschauer and E. Werle, *Biol. Chem. Hoppe-Seyler*, **356**, 1879 (1975); *b*) H. Kira, S. Hiraku and H. Terashima, *Adv. Exp. Med. Biol.*, **120**A, 237 (1979); *c*) M. Ikekita, H. Moriya, S. Ozawa and K. Kizuki, *Chem. Pharm. Bull.*, **29**, 545 (1981).
4) H. Tschesche, W. Ehret, G. Godec, C. Hirschauer, C. Kutzbach, G. Schmidt-Kastner and F. Fiedler, *Adv. Exp. Med. Biol.*, **70**, 123 (1976).
5) F. H. Carpenter, *Methods in Enzymol.*, **19**, 237 (1970).
6) M. Lemon, F. Fiedler, B. Forg-Brey, C. Hiruschauer, G. Leysath and H. Frits, *Biochem. J.*, **177**, 159 (1977).
7) K. K. Stewart and R. F. Doherty, *FEBS Lett.*, **16**, 226 (1971).
8) G. W. Schwert and Takenaka, *Biochim. Biophys. Acta*, **16**, 570 (1955).
9) F. Fiedler, B. Muller and E. Werle, *FEBS Lett.*, **24**, 41 (1972).
10) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).

11) H. Towbin, T. Staehelin and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350 (1979).
12) A. Tsugita, T. Ataka and T. Uchida, *J. Prot. Chem.*, **6**, 121 (1987).
13) H. Tscheche, G. Mair, G. Godec, F. Fiedler, W. Ehret, C. Hirschauer, M. Lemon, H. Fritz, G. Schmidt-Kastner and C. Kutzback, *Adv. Exp. Med. Biol.*, **120**A, 245 (1979).
14) F. Fiedler and H. Fritz, *Biol. Chem. Hoppe-Seyler*, **362**, 1171 (1981).
15) D. Fukushima, N. Kitamura and N. Nakanishi, *Biochemistry*, **24**, 8037 (1985).
16) S. Takahashi, A. Irie, Y. Katayama, K. Ito and Y. Miyake, *J. Biochem.* (Tokyo), **99**, 989 (1986).
17) G. H. Swift, J-C. Dagon, P. L. Ashley, S. W. Cummings and R. J. MacDonald, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 7263 (1982).
18) A. J. Mason, B. A. Evans, D. R. Cox, J. Shine and R. I. Richards, *Nature* (London), **303**, 300 (1983).
19) S. Takahashi, A. Irie, Y. Katayama, K. Ito and Y. Miyake, *Biochem. Int.*, **14**, 467 (1987).
20) R. W. Colman, Y. T. Wachtfogel, U. Kucich, G. Weinbaum, S. Hahn, R. A. Pixley, C. F. Scott, A. de Agostini, D. Burger and M. Schapira, *Blood*, **65**, 311 (1985).