# Generation of a Different Type of β-Kallikrein from Porcine Pancreatic α-Kallikrein by the Action of Chymotrypsin—Observation of Proteolytic Processing Occurring around "Kallikrein Autolysis Loop" Region

Masafumi KAMADA,*[,a] Masahiko IKEKITA,[a] Tomoko KURAHASHI,[a] Kazumasa AOKI,[a] Kazuyuki KIZUKI,[a] Hiroshi MORIYA,[a] Charles C. SWEELEY,[b] Masaharu KAMO[c] and Akira TSUGITA[c]

*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo,[a] 12, Ichigaya-Funakawara-machi, Shinjuku-ku, Tokyo 162, Japan, Department of Biochemistry, Michigan State University,[b] East Lansing, Michigan 48824, U.S.A. and Research Institute for Biosciences, Science University of Tokyo,[c] 2669, Yamazaki, Noda-shi, Chiba 278, Japan.* *Received November 28, 1989*

**The generation of a different type of β-kallikrein, designated Cβ-kallikrein, from α-kallikrein by chymotryptic action was ascertained by the following observations:**

**1) When α-kallikrein was incubated with chymotrypsin, an increase of esterolytic activity of kallikrein was observed.**

**2) In sodium dodecyl sulfate polyacrylamide gel electrophoresis, Cβ-kallikrein was found to be different from the β-kallikrein obtained from α-kallikrein by tryptic digestion, and was designated Tβ-kallikrein.**

**3) N-Terminal amino acid sequence analyses of internal light and heavy chains of Cβ-kallikrein indicated that N-termini of the light and the heavy chains were isoleucine and lysine, respectively, and that the heavy chain had most of the "kallikrein autolysis loop" sequence in its N-terminal end. In the case of Tβ-kallikrein, N-termini of the light and the heavy chains were isoleucine and alanine, respectively, and the light chain retained the "kallikrein autolysis loop" region in its C-terminal end.**

**These observations demonstrated that Cβ-kallikrein was different from the β-kallikrein prepared from autolyzed pancreas, Aβ-kallikrein, which had lost the "kallikrein autolysis loop" sequence.**

**Structural differences of the above four kallikreins (α-, Tβ-, Cβ- and Aβ-) result in somewhat different enzyme properties. The kinetic constants for the hydrolysis of synthetic substrates ($N^{\alpha}$-benzoyl-L-arginine ethyl ester and $N^{\alpha}$-tosyl-L-arginine methyl ester) of these kallikreins differed from each other, and inhibitory profiles against $\alpha_1$-antitrypsin were also different. These observations suggest that the "kallikrein autolysis loop" region may play an important role in the regulation of kallikrein activity *in vivo*.**

**Keywords** tissue kallikrein; porcine pancreatic kallikrein; prokallikrein; activation mechanism; α-kallikrein; β-kallikrein; proteolytic processing; kallikrein autolysis loop; structure–activity relationship

## Introduction

In a previous paper, the generation of α- and β-kallikreins from porcine pancreatic prokallikrein by tryptic action was demonstrated.[1] Initially, prokallikrein was activated to α-kallikrein by trypsin, then α-kallikrein was further converted to the Tβ-kallikrein with decreasing esterolytic activity. Tβ-kallikrein was different from Aβ-kallikrein obtained from autolyzed pancreas. Among our observations on the serial activation of prokallikrein, incubation of α-kallikrein with chymotrypsin caused an increase of esterolytic activity of kallikrein. This phenomenon was different from that obsrerved when α-kallikrein was incubated with trypsin, and suggested that another different type of β-kallikrein is generated from α-kallikrein by chymotryptic action. Characterization of this Cβ-kallikrein has been carried out, and the generation of active kallikreins from prokallikrein by serial proteolytic processing is also demonstrated. The enzymatic properties and structural difference of these active kallikreins have also been investigated, and structure–activity relationships among these generated active kallikreins are discussed.

## Materials and Methods

**Materials** $N^{\alpha}$-Benzoyl-L-arginine ethyl ester (Bz–Arg–OEt) and $N^{\alpha}$-tosyl-L-arginine methyl ester (Tos–Arg–OMe) were purchased from Peptide Institute, Inc. (Osaka, Japan). TLCK (*p*-toluenesulfonyl-L-lysine chloromethyl ketone hydrochloride) treated α-chymotrypsin (type VII) and human $\alpha_1$-antitrypsin were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Trypsin from porcine pancreas (Biozyme Lab. Ltd., South Wales, Great Britain) was used after treatment with L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK).[2] Molecular mass marker proteins from Pharmacia LKB Biotechnology (Uppsala, Sweden) were also used. All the reagents for sequence analysis were obtained from Applied Biosystems (ABI, Foster City, Ca., U.S.A.). Polyvinylidene difluoride membrane (PVDF, Immobilon Transfer) was purchased from Millipore (Bedford, Ma., U.S.A.). Kallikrein B′′ (82.3 EU/$A_{280}$) as α-kallikrein and Kallikrein B′′-T (35 EU/$A_{280}$) as Tβ-kallikrein in the present paper were prepared as described previously.[1] Kallikrein B as Aβ-kallikrein in the present paper and other reagents were also the same as described in our previous paper.[1]

**Bz–Arg–OEt Esterolytic Activity Assay** Bz–Arg–OEt esterolytic activities of kallikrein and trypsin were analyzed spectrophotometrically according to the method previously described.[1] The final substrate concentrations of Bz–Arg–OEt used were 1 mM for α- and Tβ-kallikreins and 0.5 mM for Aβ- and Cβ-kallikreins. 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.

**Active Site Titrations** The active enzyme concentrations of kallikrein and trypsin were determined by titration with *p*-nitrophenyl-*p*′-guanidinobenzoate according to the method of Fiedler *et al.*[3]

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)** Gel electrophoresis on slabs was performed as described by Laemmli (gel concentration: 12.5%).[4] After the electrophoresis, the gels were stained with silver using an Ag-stain kit (Daiichi Pure Chemical Co., Ltd., Tokyo, Japan).

**Analysis of N-Terminal Amino Acid Sequence** Automated analysis of the N-terminal sequence of proteins was performed with a pulse liquid protein sequencer (Model 477A protein sequencer equipped with a 120A PTH Analyzer, ABI).

**Sequencing from Proteins Electroblotted onto PVDF Membranes** Electroblotting was performed according to the method of Matsudaira.[5] After SDS-PAGE of reduced kallikrein, about 200 pmol, the protein bands were electrically blotted onto the PVDF membrane. The proteins were stained with Coomassie Brilliant Blue R-250 and the bands were cut out with a clean razor. Then, the membrane was set on the protein sequencer, and N-terminal sequence analysis was carried out.

**Analysis of C-Terminal Amino Acid of Cβ-Kallikrein** Cβ-Kallikrein

(400 pmol) was dissolved in 50 μl of 0.1 M pyridine–acetate–collidine buffer (pH 8.3). Then, 2.5 μl of carboxypeptidase (CPase) A solution (2.5 mg/ml) was added to the kallikrein solution and the mixture was incubated at 37 °C. After incubation, 50 μl of 70% formic acid was added to the mixture to stop the reaction. The liberated amino acids were analyzed with an Irika amino acid analyzer, Model A-8700 (Irika Instruments, Inc., Kyoto, Japan).

## Results and Discussion

**Digestion of α-Kallikrein with Chymotrypsin** Alpha-kallikrein was incubated with chymotrypsin at 37 °C, pH 7.0, for various periods of time and the remaining esterolytic activities were measured. As shown in Fig. 1, the esterolytic activity gradually increased with increasing incubation time and reached a plateau at 24 h corresponding to about 150% of the initial activity. This phenomenon was different from that observed when α-kallikrein was incubated with trypsin.[1] In that case, the esterolytic activity decreased to 60% of the initial activity, and Tβ-kallikrein was generated. These observations suggested that a different type of β-kallikrein (designated Cβ-kallikrein) could be generated from α-kallikrein by this procedure.

The following approach was used to remove chymotrypsin from Cβ-kallikrein in the reaction mixture. After the mixture was dialyzed against 5 mS ammonium acetate solution (pH 6.0), the dialyzate was applied to a CM-Cellulofine column (1 × 5 cm). Chymotrypsin was adsorbed to the column and Cβ-kallikrein eluted in the non-adsorbed fraction. This fraction was applied to a DEAE-Sepharose CL-6B column (1 × 5 cm); after the column was washed with 20 ml of 18 mS ammonium acetate (pH 6.0), the absorbed Cβ-kallikrein was eluted with 35 mS ammonium acetate solution (pH 6.0). The eluted fractions which showed esterolytic activity of Cβ-kallikrein were pooled, the Cβ-kallikrein fraction was lyophilized after dialysis against purified water, and the powder stored at −20 °C until use.

**Peptide Chain Sequence of Cβ-Kallikrein** Figure 2-C shows SDS-PAGE of Cβ-kallikrein, as compared with α- and Tβ-kallikreins (Fig. 2-A, B). The obtained Cβ-kallikrein was observed to consist of two bands, molecular mass variants, 36 and 33 kDa like α- and Tβ-kallikreins under non-reducing conditions. Under reducing conditions, in contrast, Cβ-kallikrein showed three bands of 30.5, 26.5 and 14.4 kDa. Both N-terminal sequences of 30.5 and 26.5 kDa bands were identical, Lys–X–His–Thr–Lys–Ala–Asp–Gly–Lys–Asp–, whereas that of the sequence of 14.4 kDa band was Ile–Ile–Gly–Gly–Arg–Glu–X–Glu–; this latter sequence was identical with those of α-kallikrein and of the light chain of Tβ-kallikrein. These results indicated that the Cβ-kallikrein consists of a light chain (located in the N-terminal end of the molecule) of 14.4 kDa and two types of heavy chains (located in the C-terminal end of the molecule) of 30.5 and 26.5 kDa, molecular mass variants that might be different in the amount of attached carbohydrate. As shown in Fig. 2-B, Tβ-kallikrein consists of two types of light chains of 17.5 and 14.4 kDa, also presumed to be molecular mass variants, and one heavy chain of 25.5 kDa. Cβ-Kallikrein is therefore proposed to

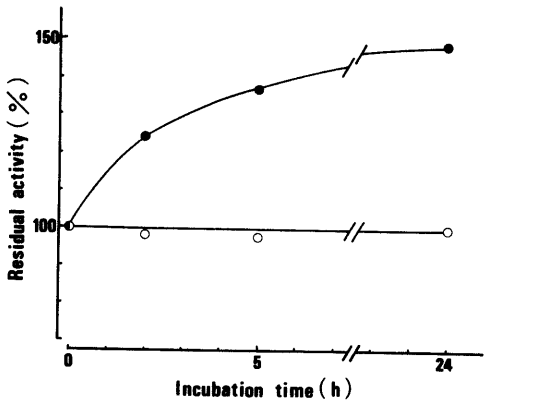


Fig. 1. Effect of Chymotrypsin on the Esterolytic Activity of α-Kallikrein

Purified α-kallikrein solution (60 EU in 1.3 ml of 0.1 M triethanolamine–HCl buffer containing 20 mM $CaCl_2$, pH 7.0) and 1 ml of chymotrypsin solution (230 μg/ml in the same buffer) were mixed and incubated at 37 °C. After the indicated incubation period, aliquots (25 μl) were taken from the incubation mixture. The esterolytic activity of the mixture toward Bz–Arg–OEt was measured. The esterolytic activity of α-kallikrein before treatment with chymotrypsin was taken as 100%. The symbols, (●) and (○) indicate treatment with and without chymotrypsin, respectively.

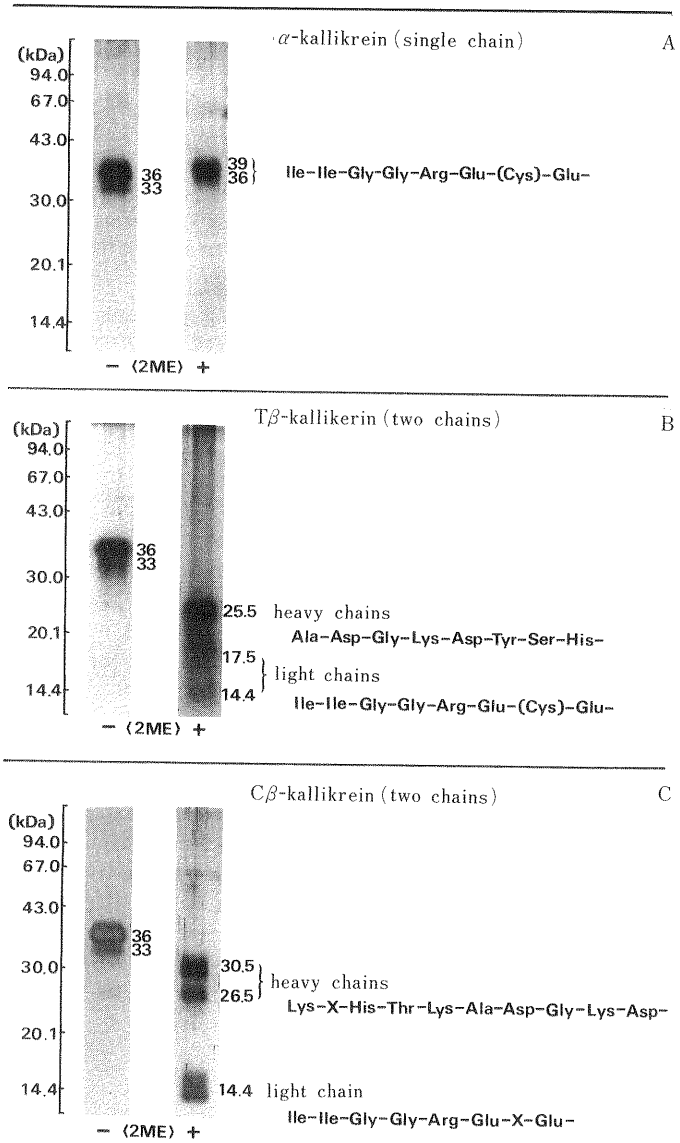


Fig. 2. SDS-PAGE Analyses of α-, Tβ- and Cβ-Kallikreins

Each 20 pmol of sample was treated with (+) or without (−) 2-mercaptoethanol (2ME) and applied on the gel. After electrophoresis, the gels were stained with silver. N-Terminal sequences of the bands of Cβ-kallikrein were determined by the electroblotting described in Materials and Methods. Those of α- and Tβ-kallikreins were assigned by the results described previously.[1]

be a β-type kallikrein but different from Tβ-kallikrein.

**Assignment of the Amino Acid Sequence of "Kallikrein Autolysis Loop" Region** Difference between the N-terminal sequences of both heavy chains of Cβ- and Tβ-kallikreins indicated that Cβ-kallikrein had an additional pentapeptide, Lys–X–His–Thr–Lys at its N-terminal end. This peptide sequence was not found in the primary sequence of Aβ-kallikrein derived from autolyzed pancreas.[6] Since Aβ-kallikrein loses the kallikrein autolysis loop sequence which was previously unknown but considered to consist of several amino acids,[7] the above pentapeptide sequence presumably accounts for a large part of the kallikrein autolysis loop region.

Upon C-terminal sequence analysis of the native Cβ-kallikrein (400 pmol) using CPase A (pH 8.3, 37 °C), Leu (80 pmol) was observed after 30 min incubation, and Leu (307 pmol) and Ser (95 pmol) were detected after 3 h incubation. CPase A cannot release C-terminal proline which is anticipated to be the C-terminus of the heavy chain of Cβ-kallikrein (see Fig. 4). Therefore, CPase A could release amino acids only from the C-terminus of the light chain. The C-terminal sequence of the light chain was concluded to be –Ser–Leu from these observations.

When liberation of free amino acids during the chymotryptic generation of Cβ-kallikrein from α-kallikrein (1 nmol) was investigated, Leu (1.09 nmol) was significantly detected, thus indicating that one leucine residue is liberated from α-kallikrein molecule in this process.

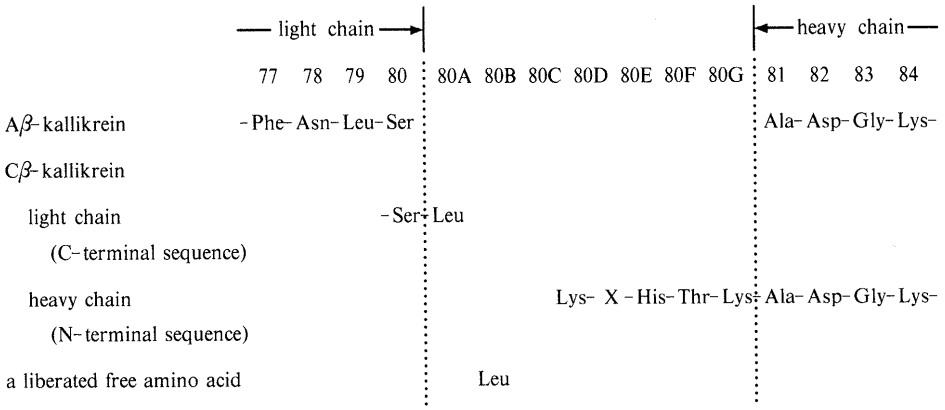


Fig. 3. Assignment of N- and C-Terminal Sequences of Cβ-Kallikrein in the "Kallikrein Autolysis Loop" Region of Aβ-Kallikrein

The numbering is from the N-terminal amino acid of the light chain to the C-terminal amino acid of the heavy chain of Aβ-kallikrein. The kallikrein autolysis loop region locates between C-terminal $^{80}$Ser of the light chain and N-terminal $^{81}$Ala of the heavy chain in the sequence of Aβ-kallikrein. $^{80B}$Leu is a free amino acid liberated during chymotryptic generation of Cβ-kallikrein.

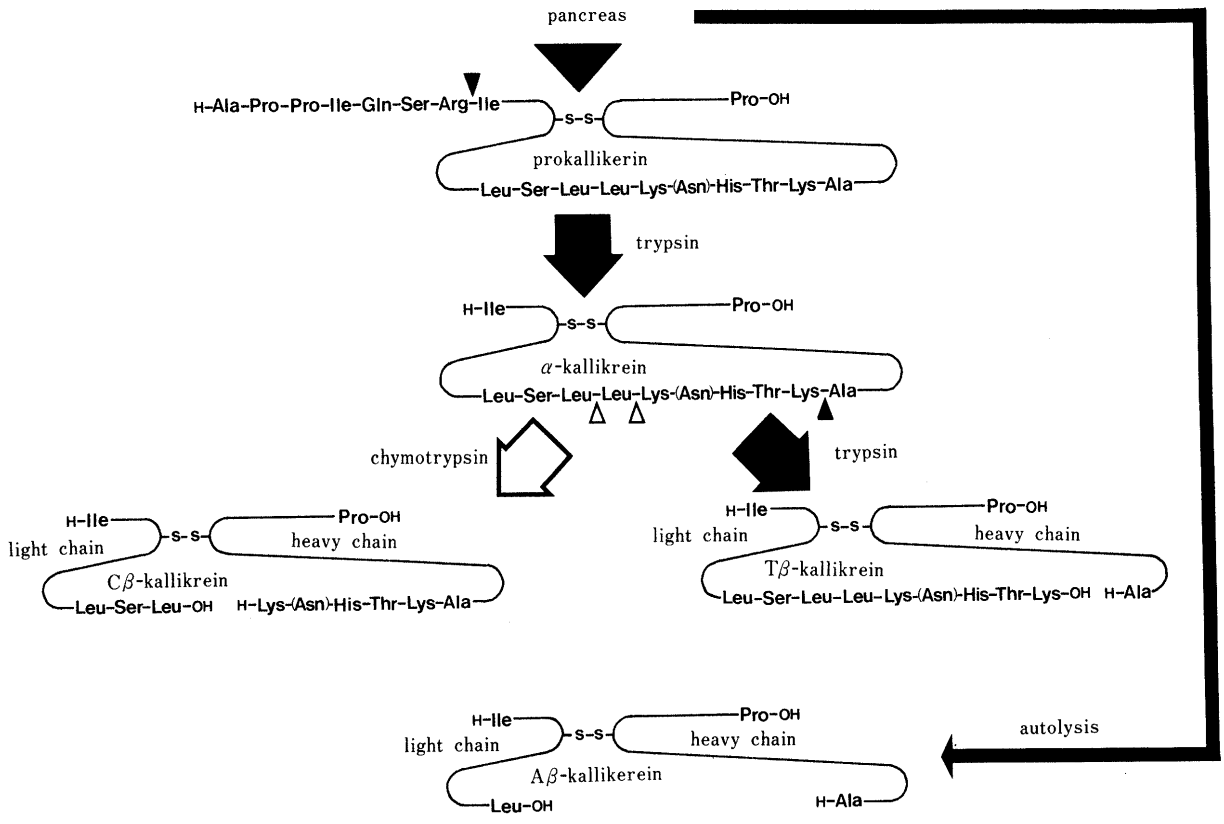


Fig. 4. Assigned Polypeptide Chain Structure Relationship among Porcine Pancreatic Prokallikrein and α- and β-Kallikreins

The N-terminal heptapeptide of prokallikrein, Ala–Pro–Pro–Ile–Gln–Ser–Arg is so-called propeptide. Arrowheads indicate the sites of tryptic (▲) and chymotryptic (△) cleavages. Undetermined amino acid is indicated in parentheses.

N- and C-terminal sequences of Cβ-kallikrein and a free leucine residue were therefore located near the kallikrein autolysis loop sequence of Aβ-kallikrein (Fig. 3). This sequence locates between $^{80}$Ser residue, the C-terminus of the light chain, and $^{81}$Ala residue, the N-terminus of the heavy chain in the primary sequence of Aβ-kallikrein. Fiedler and Fritz reported that the C-terminal $^{80}$Ser was present in about 50% of Aβ-kallikrein, with the remainder being $^{79}$Leu.[7] Thus, seven amino acid residues must be inserted between $^{80}$Ser and $^{81}$Ala. In the generation process of Cβ-kallikrein from α-kallikrein, both the $^{80A}$Leu-$^{80B}$Leu and the $^{80B}$Leu-$^{80C}$Lys bonds may be the cleavage sites with chymotrypsin. The primary sequence of human and rat pancreatic kallikreins predicted from their nucleotide sequences were previously reported by Fukushima *et al.*[8] and Swift *et al.*,[9] respectively. The kallikrein autolysis loop sequences of human and rat pancreatic kallikreins which correspond to the heptapeptide sequence between $^{80A}$Leu and $^{80G}$Lys were Leu–Leu–Glu–Asn–His–Thr–Arg and Leu–Ile–Trp–Asn–His–Thr–Arg, respectively. In the present study, the amino acid residue in position 80D was not identified by the sequencer; a possible amino acid residue is glycosylated asparagine from the fitness of the sequence, Asn–X–Thr which is a consensus glycosylation sequence,[10] and the homology of the conserved sequence, Asn–His–Thr, in the kallikrein autolysis loop regions of human and rat pancreatic kallikreins. Thus, the heptapeptide sequence, Leu–Leu–Lys–(Asn)–His–Thr–Lys is proposed to be the sequence of the kallikrein autolysis loop region of porcine pancreatic kallikrein.

**Peptide Chain Structure Relationship among Prokallikrein and α- and β-Kallikreins** Activation of prokallikrein to α-kallikrein and conversion of α-kallikrein to Tβ-kallikrein by tryptic action was demonstrated previously.[1] In the present study, conversion of α-kallikrein to Cβ-kallikrein by the action of chymotrypsin has been demonstrated. The assigned peptide chain sequences of prokallikrein and the generated kallikreins are summarized in Fig. 4.

After activation of prokallikrein to α-kallikrein by the action of trypsin (the Arg–Ile bond is the tryptic cleavage site with simultaneous release of the N-terminal heptapeptide), α-kallikrein is further converted to Tβ-kallikrein by tryptic cleavage of the Lys–Ala bond in the kallikrein autolysis loop region, and also converted to Cβ-kallikrein by chymotryptic cleavage of both the Leu–Leu and the Leu–Lys bonds in the same region. This would account for the fact that the heavy chain of Cβ-kallikrein retains most of the kallikrein autolysis loop region in its N-terminal end. On the other hand, in Tβ-kallikrein, the light chain has the kallikrein autolysis loop sequence in its C-terminal end. This clearly demonstrates the difference between Cβ-kallikrein and Tβ-kallikrein. Cβ-kallikrein is also different from Aβ-kallikrein, in which the kallikrein autolysis loop region is lost. The peptide chain sequence of our Aβ-kallikrein, obtained from autolyzed pancreas, had previously been assigned as shown in Fig. 4. The C-terminal amino acid residue of the light chain of this protein is leucine but not serine.[1]

The mechanism of the artificial autolysis process of prokallikrein is still obscure, but trypsin, chymotrypsin and CPase A possibly act in the generation process, based on the observations in the present study. After initial activation of prokallikrein to α-kallikrein, proteolytic processing of active kallikrein molecules takes place around the kallikrein autolysis loop region and results in the generation of three different types of β-kallikrein.

TABLE I. Kinetic Constants for the Hydrolysis of Bz–Arg–OEt and Tos–Arg–OMe by α-, Tβ-, Cβ- and Aβ-Kallikreins

| | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}s^{-1}$) |
|---|---|---|---|
| Bz–Arg–OEt | | | |
| α-Kallikrein[a] | 0.234±0.007 | 62.5±1.2 | 287 |
| Tβ-Kallikrein[a] | 0.333±0.023 | 53.8±2.5 | 162 |
| Cβ-Kallikrein | 0.080±0.002 | 99.6±1.5 | 1245 |
| Aβ-Kallikrein[a] | 0.109±0.005 | 81.6±2.3 | 749 |
| Tos–Arg–OMe | | | |
| α-Kallikrein | 0.039±0.002 | 3.13±0.04 | 80.3 |
| Tβ-Kallikrein | 0.117±0.007 | 9.38±0.22 | 80.2 |
| Cβ-Kallikrein | 0.022±0.001 | 2.35±0.04 | 115 |
| Aβ-Kallikrein | 0.048±0.002 | 2.75±0.19 | 57.3 |

$K_m$ and $k_{cat}$ values of the four kallikreins were estimated at concentrations of Bz–Arg–OEt, 0.05—1.0 mM and of Tos–Arg–OMe, 0.05—0.5 mM (pH 8.0, 25 °C). Values of $K_m$ and $k_{cat}$ are means±S.E. for three determinations. *a*) Previously reported data.[1]

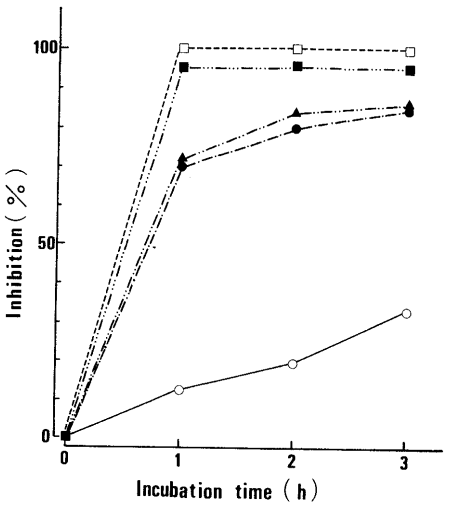


Fig. 5. Inhibition with $\alpha_1$-Antitrypsin for α-, Tβ-, Cβ- and Aβ-Kallikreins and Trypsin

Each 0.1 ml of α-, Tβ-, Cβ- and Aβ-kallikreins and trypsin solutions (1 nmol/ml each) and 0.1 ml of $\alpha_1$-antitrypsin solution (3 mg/ml in 0.1 M triethanolamine–HCl buffer containing 20 mM $CaCl_2$, pH 7.0) were mixed and incubated at 37 °C. After the indicated time, each mixture was transferred to an ice-bath. Residual esterolytic activity of the mixture was then measured immediately. The result was expressed as per cent inhibition of the esterolytic activity in the absence of the inhibitor. ○, α-kallikrein; ●, Tβ-kallikrein; ▲, Cβ-kallikrein; ■, Aβ-kallikrein; □, trypsin.

**Kinetic Constants for Hydrolysis of Synthetic Substrates by Kallikreins** Kinetic constants for the hydrolysis of the synthetic substrates, Bz–Arg–OEt and Tos–Arg–OMe, with α-, Tβ-, Cβ- and Aβ-kallikreins are shown in Table I; as shown, they differed from each other. These results suggested that the cleavages of the Lys–Ala bond, generation of Tβ-kallikrein and both the Leu–Leu and the Leu–Lys bonds, the generation of Cβ-kallikrein, and the release of the kallikrein autolysis loop, the generation of Aβ-kallikrein had marked influence on the kinetics of α-kallikrein.

**Inhibition of Kallikreins by $\alpha_1$-Antitrypsin** Figure 5 shows the inhibitory profiles of α-, Tβ-, Cβ- and Aβ-kallikreins and trypsin with $\alpha_1$-antitrypsin (an inhibitor *in vivo*). Alpha-kallikrein was weakly inhibited by $\alpha_1$-antitrypsin (13 and 33% inhibition after 1 h and 3 h incu-

bations, respectively). On the other hand, three β-kallikreins (Tβ-, Cβ- and Aβ-) were largely inhibited (up to 70% inhibition at 1 h incubation and up to 85% inhibition at 3 h incubation), while trypsin was completely inhibited by $\alpha_1$-antitrypsin. These results clearly showed quite a different inhibitory effect of $\alpha_1$-antitrypsin on α- and β-kallikreins.

In the present study, we have demonstrated that proteolytic processing of α-kallikrein by trypsin and chymotrypsin occurred around the kallikrein autolysis loop region after initial activation of prokallikrein to α-kallikrein. It is noteworthy that the conversion of α- to β-kallikreins alters not only the kinetic profiles toward synthetic substrates but also the inhibitory effects against $\alpha_1$-antitrypsin in our *in vitro* systems. Therefore, it would be very interesting to investigate the process of protease activation of tissue prokallikrein *in vivo*.

**Acknowledgement** This work was supported by grants from the Ministry of Education, Science and Culture of Japan and the Sankyo Foundation of Life Science, Japan.

**References**

1) M. Kamada, K. Aoki, M. Ikekita, K. Kizuki, H. Moriya, M. Kamo and A. Tsugita, *Chem. Pharm. Bull.*, **36**, 4891 (1988).
2) F. H. Carpenter, *Methods in Enzymol.*, **19**, 237 (1970).
3) F. Fiedler, B. Müller and E. Werle, *FEBS Lett.*, **24**, 41 (1972).
4) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
5) P. Matsudaira, *J. Biol. Chem.*, **262**, 10035 (1987).
6) H. Tschesche, 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**, 245 (1979).
7) F. Fiedler and H. Fritz, *Biochem. Hoppe-Seyler*, **362**, 1171 (1981).
8) D. Fukushima, N. Kitamura and N. Nakanishi, *Biochemistry*, **24**, 8037 (1985).
9) 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).
10) E. Bause, *Biochem. J.*, **209**, 331 (1983).