Purification and Characterization of Rat T-kininogens Isolated from Plasma of Adjuvant-treated Rats

IDENTIFICATION OF THREE KINDS OF T-KININOGENS

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Kei-ichi Enjojï†, Hisao Kato‡, Izumi Hayashi†, Sachiko Oh-ishï, and Sadaaki Iwanaga

From the Department of Biology, Faculty of Science, Kyushu University, 33 Higashi-ku, Fukuoka 812 and the Department of Pharmacology, School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan

Two T-kininogens (TI- and TII-kininogens) found in plasma of Freund’s adjuvant-treated rats were purified by several chromatographic procedures. The isolated TI- and TII-kininogens showed different mobilities on polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate, but were indistinguishable in the presence of sodium dodecyl sulfate. They were also indistinguishable in amino acid composition and antigenicity, but differed in sialic acid content. The NH2- and COOH-terminal sequences were determined. In the 30 NH2-terminal residues, 2 were different. The kinin regions in the COOH-terminal portions of the two kininogens have sequences that demonstrate TI-kininogen contains a mixture of two kinin-containing regions, with substitution of 4 amino acid residues, one of which is identical to the COOH-terminal portion of α1-major acute phase protein (Cole, T., Inglis, A. S., Roxburgh, C. M., Howlett, G. J., and Schreiber, G. (1985) FEBS Lett. 182, 57–61) and the other to the COOH-terminal portion of TII-kininogen predicted from cDNA sequences. The amino acid sequence of the kinin-containing region from TI-kininogen is the same as the COOH-terminal portion of TII-kininogen predicted from the cDNA. These results indicate that T-kininogens from the plasma of adjuvant-treated rats consist of a family of kininogens, that is, TI- and TII-kininogens (separable on DEAE-Sephadex A-50), and that TII-kininogen consists of at least two variants (TIα and TIβ) which correspond to the α1-major acute phase protein reported by Cole et al. and TI-kininogen reported by Furuto-Kato et al., respectively. Immunoblotting studies with plasmas from non-inflamed and adjuvant-treated rats also indicate that T-kininogen which was previously isolated from non-inflamed rat plasma corresponds to TI-kininogen and that T-II-kininogen is newly generated after treatment of rats with adjuvant.

T-kininogen, one of the low M, kininogens in rat plasma, is a precursor of T-kinin (Ile-Ser-bradykinin), which can be formed by action of excess trypsin (1). We previously isolated two low M, kininogens (tentatively named low M, kininogens A and B) from Sprague-Dawley rat plasma and demonstrated that one of them, low M, kininogen A, is resistant to kallikreins and corresponds to T-kininogen and that the second, low M, kininogen B, has the properties of low M, kininogens from bovine and human plasma (9). Furuto-Kato et al. (2) have presented evidence that there exist two kinds of mRNAs for T-kininogens, TI- and TII-kininogens, in non-inflamed rat liver; and Kageyama et al. (3) found an increase of mRNA levels for both T-kininogens after injection of bacterial endotoxin into Wistar rats. Recently, the amino acid sequence of α1-major acute phase protein (MAP),1 which is found in plasma from rats treated with turpentine, was reported by two groups of investigators (4–6). An increase of T-kininogen content in rat plasma after the injection of Freund’s adjuvant was also found by Barlas et al. (7).

Attention has been focused on the role of T-kininogen in inflammation, not only because its content in plasma is dramatically increased after the induction of inflammation, but also because T-kininogen has strong inhibitory activity on thiol proteinases, such as papain and rat cathepsins B, H, and L (8). In the previous paper (9), we have demonstrated that T-kininogen from the plasma of non-inflamed rats is a mixture of at least two kinds of T-kininogens with slightly different amino acid sequences and that TI-kininogen is not present in significant amounts. Okamoto and Greenbaum (24) found that the T-kininogen in plasma of rats inflamed with Freund’s adjuvant can be separated into two components on DEAE-Sephadex A-50. However, these kininogens have not been characterized.

In this present study, we isolated two T-kininogens (TI and TII) from Freund’s adjuvant-treated rat plasma and examined their NH2- and COOH-terminal sequences. We present evidence that TII-kininogen corresponds to the TII-kininogen reported by Furuto-Kato et al. (2), but that TI-kininogen consists of two kininogens, one of which corresponds to the MAP reported by Cole et al. (5) and the second of which corresponds to the TI-kininogen reported by Furuto-Kato et al. (2). We will also demonstrate that TII-kininogen is generated in the adjuvant-treated rat.

The abbreviations used are: MAP, α1-major acute phase protein; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; HPLC, high performance liquid chromatography; PE, S-pyridylethylated; PTH, phenylthiobantoin; PPLC, fast protein liquid chromatography; DFP, diisopropyl fluorophosphate.

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† Present address: National Cardiovascular Center, Research Institute, Fujishiro-dai 5 chome, Suita, Osaka 565, Japan.

‡ To whom correspondence should be addressed.
Experimental Procedures

Results

Purification of T-kininogens from Plasma of Adjuvant-treated Rats—Purification procedures for TI- and TII-kininogens are summarized in Fig. 1. One-hundred ml of plasma from rats treated with adjuvant as described under “Experimental Procedures” was applied to a column of DEAE-Sephadex A-50. Protein was eluted by a linear salt gradient as described for Fig. 2. The kinin activity was found as three peaks (Fig. 2). The third peak showed the ability to correct the prolonged clotting time of Fitzgerald-trait plasma, indicating that this peak contains high M, kininogen (11). The first two peaks, designated as TI- and TII-kininogens, respectively, were subjected to further purification. The pooled fractions in Fig. 2 were applied to a column (5.5 x 11 cm) of zinc-chelation Sepharose 6B, and the nonadsorbed fractions were dialyzed overnight against 0.05 m sodium phosphate buffer, pH 6.3, containing Polybrene (50 mg/liter) and benzamidine (0.5 g/liter). Each dialysate was applied to a column (4.5 x 21 cm) of CM-Sephadex C-50 equilibrated with 0.05 m sodium acetate buffer, pH 6.3, containing Polybrene and benzamidine; and the nonadsorbed fractions were lyophilized. Low M, kininogen B was removed at this step since it is adsorbed on the column (9). The lyophilized materials were dissolved in 10 ml of distilled water and applied to a column of Sephadex G-150. Fig. 3 (A and B) shows the elution profiles of the TI- and TII-kininogen fractions. The kininogens were eluted in the second protein peak. Each of the kininogen fractions was applied to a column (3.1 x 7.2 cm) of hydroxyapatite equilibrated with 0.01 m sodium phosphate buffer, pH 7.8. TI- and TII-kininogens were found in the nonadsorbed fraction (data not shown). TII-kininogen was further subjected to hydrophobic chromatography on a column of phenyl-5PW and was eluted as shown in Fig. 4. Kinin releasing activity was found throughout the protein peak. On SDS-PAGE in the presence of 2-mercaptoethanol, fractions 21-33 gave two bands, one major and another minor, on disc PAGE, as shown in Fig. 5A (lanes 1 and 2). These bands overlapped, indicating that the preparations were cross-contaminated. To separate TI- and TII-kininogens completely, each fraction (TI-kininogen from the hydroxyapatite column and TII-kininogen from the phenyl-5PW column) was applied separately to a column of DEAE-Sephadex A-50. As shown in Fig. 6, on elution by a linear salt gradient to 0.2 m NaCl, TI-kininogen appeared faster than TII-kininogen; and both kininogens when pooled as indicated by the solid bars (Fig. 6) showed a single band on electrophoresis (Fig. 5B, lanes 2 and 3). The yields of T-kininogens thus isolated were calculated to be 13.7 for TI-kininogen and 6.8 for TII-kininogen in terms of absorbance units at 280 nm. A mixture of TI- and TII-kininogens was resolved into two bands (Fig. 5B, lane 4). The mobility of the T-kininogen from plasma of non-inflamed rats on disc PAGE corresponded to that of TI-kininogen (Fig. 5B, lane 1).

Sialidase Treatment of TI- and TII-kininogens—After treatment of TI- and TII-kininogens with sialidase, their mobilities on disc PAGE became slower than those of the native kininogens, as shown in Fig. 5C. However, the mobilities of the treated TI- and TII-kininogens still differed (Fig. 5C, lanes 2 and 3). This result indicates that the different mobilities of the TI- and TII-kininogens may not be simply due to different contents of sialic acid. The mobility of sialidase-treated T-kininogen from non-inflamed rat plasma corresponds to that of sialidase-treated TI-kininogen (Fig. 5C, lane 1). Fig. 5C also shows microheterogeneity in these three kininogens (T, TI, and TII) because they gave several bands with slightly different mobilities. In spite of these heterogeneities, the mobilities of the three kininogens on SDS-PAGE in the absence or presence of 2-mercaptoethanol were indistinguishable (Fig. 7).

Properties of TI- and TII-kininogens—Table I shows the amino acid composition and sialic acid content of TI- and TII-kininogen in comparison with T-kininogen from plasma of non-inflamed rats and MAP. The values for TI- and TII-kininogens are indistinguishable from those of T-kininogen and MAP. The sialic acid content of TII-kininogen is higher than that of TI-kininogen and comparable to that of MAP. The sialic acid content of TI-kininogen is comparable to T-kininogen from the non-inflamed rat.

Table II shows the NH2-terminal sequences of TI- and TII-kininogens. Since no PTH amino acids were detected from the S-pyridylethylated kininogens, these sequences were determined after treatment with pyroglutamate aminopeptidase. Residues detected up to step 29 were the same except for steps 7 and 8.

Amino Acid Sequences of the COOH-terminal Regions of the Kinin Moiety in TI- and TII-kininogens—The S-pyridylethylated TI- and TII-kininogens were treated with cyanogen bromide, and the digests were applied to columns (1.6 x 145 cm) of Sephadex G-75 equilibrated with 10% acetic acid. The kinin activity after trypsin treatment was found as a single peak in a low molecular weight fraction. Each fraction from TI- and TII-kininogens was pooled and applied to a reversed-phase HPLC column. As shown in Fig. 8, the kinin-containing regions derived from TI- and TII-kininogens were found in a single peak (cross-hatched peak). Sequence analysis gave the results summarized in Fig. 10. The sequence of the kinin-containing region from TII-kininogen was determined through 50 steps (except for step 49). Two PTH amino acids were found at steps 39 and 45.
Rat Plasma T-kininogens and Acute Phase Proteins

Fig. 2. DEAE-Sephadex A-50 chromatography of plasma from adjuvant-treated rats. To a column (4.5 x 18.5 cm) of DEAE-Sephadex A-50 equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.05 M NaCl, 1 mM EDTA, Polybrene (50 mg/liter), and benzamidine (0.5 g/liter), 100 ml of plasma which was dialyzed against the equilibration buffer for 1 day was applied. After washing the column with the equilibration buffer without EDTA, protein was eluted by a linear gradient formed with 2 liters each of the washing buffer and buffer containing 0.3 M NaCl. Arrows indicate the fractions at which gradient elution and stepwise elution with 0.5 M NaCl were started. Kinin activity was measured by contraction of rat uterus after incubation of each fraction with snake venom kininogenase as described under "Experimental Procedures" (○-○-○). The activity of high M₉, kininogen was measured by correction of the prolonged clotting time of Fitzgerald-trait plasma (O--O). The kininogen fractions were pooled as indicated by the solid bars and are designated TI- and TII-kininogens. KGN, kininogen; BK, bradykinin.

Fig. 5. Disc PAGE of T-kininogens. A: lane 1, TI-kininogen from hydroxylapatite column chromatography; lane 2, TII-kininogen from phenyl-5PW column chromatography. B: lane 1, T-kininogen from plasma of non-inflamed rats; lane 2, TI-kininogen; lane 3, TII-kininogen; lane 4, mixture of TI- and TII-kininogens. C: T-kininogens in B were treated with sialidase as described under "Experimental Procedures."

containing region from TII-kininogen was determined up to step 53 (except for step 52).

To determine the complete sequence of the kinin-containing regions from TI- and TII-kininogens, the peptides were hydrolyzed with lysyl-endopeptidase, and the digests were separately subjected to reversed-phase HPLC. As shown in Fig. 9, five peptides were isolated from each digest, and their amino acid compositions were determined. Fig. 10 shows the sequences of these five peptides. Fragments L-2 and L-3 from TI-kininogen were found to be analogous peptides with substitutions at residues 39, 45, 46, and 55. This result indicates that the kinin-containing region from TI-kininogen is a mixture of two closely related sequences.

Fig. 6. DEAE-Sephadex A-50 chromatography of TI- and TII-kininogens. TI-kininogen from hydroxylapatite or TII-kininogen from phenyl-5PW was applied to a column (0.55 x 8.2 cm) of DEAE-Sephadex A-50 equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.05 M NaCl, benzamidine (0.5 g/liter), and Polybrene (50 mg/liter). After washing the column, protein was eluted by a linear gradient as shown using an FPLC system. A, TI-kininogen from hydroxylapatite. B, TII-kininogen from phenyl-5PW chromatography. Kininogen fractions were pooled as indicated by the solid bars.
Fig. 7. SDS-PAGE of T-kininogens. Lane 1, T-kininogen from non-inflamed rat plasma; lane 2, TI-kininogen; lane 3, TII-kininogen.

Fig. 10 summarizes the amino acid sequences of the kinin-containing regions from TI- and TII-kininogens. They consist of four peptides, L-5, L-4, L-1, and L-2. The overlapping of these fragments was confirmed by Edman degradation of the parent fragments. Fragment L-3 from TI-kininogen is analogous to L-2 with substitution of 4 amino acid residues. Since fragment L-3 contains galactosamine and the PTH amino acid at step 15 was not identified, threonine 15 is assumed to have attached carbohydrate. L-3 from TII-kininogen has the same amino acid composition as L-2. Galactosamine was found in L-2 and L-3. Since no PTH amino acid was detected at steps 15 of L-2 and L-3, carbohydrate is assumed to be attached at threonine 15 in both L-2 and L-3. These results demonstrate that the kinin-containing region from TI-kininogen consists of two heterogeneous sequences, whereas the kinin-containing region from TII-kininogen consists of a single sequence which may be heterologous in carbohydrate content.

Immunoblotting of TI- and TII-kininogens in Plasma—Fig. 11 (A and B) shows immunodiffusion of TI- and TII-kininogens with antisera against TI-kininogen and high M4, kininogen from non-inflamed rat plasma. TI- and TII-kininogens gave a fused precipitin line with each other and with T-kininogen against T-kininogen antiserum. However, they did not cross-react with antisera against high M4, kininogen, as did T-kininogen. Fig. 11C shows immunoblots of T-kininogens in plasma from non-inflamed and adjuvant-treated rats. The plasma from two rats was subjected to polyacrylamide gel electrophoresis and immunoblotted using anti-T-kininogen antiserum, as described under "Experimental Procedures." T-kininogen in non-inflamed plasma (Fig. 11C, lanes 1, and 2) gave a single band with the same mobility as TI-kininogen (Fig. 11C, lane 5). By contrast, T-kininogens in plasma from adjuvant-treated rats (Fig. 11C, lanes 3 and 4) gave two bands which corresponded to TI- and TII-kininogens (Fig. 11C, lanes 5 and 6). Several faint bands with slower mobilities are believed to be polymers of T-kininogen probably produced by freezing and thawing. The amounts of T-kininogen loaded were calculated to be about 0.4 μg in lanes 1 and 2, calculating from the plasma level of T-kininogen in non-inflamed rat plasma (25). The amounts of T-kininogen loaded in lanes 3 and 4 are presumed to be more than 10-fold higher than in lanes 1 and 2 since the plasma level of T-kininogen in inflamed rat plasma increases about 10-20-fold. Although the amounts of T-kininogen loaded were different in plasmas from non-inflamed and inflamed rats, the results indicate that the T-II kininogen content is significantly lower in non-inflamed rat plasma. This result confirms the previous results (9) that only TI-kininogen is present in significant amounts in non-inflamed rat plasma and demonstrates that TII-kininogen appears on adjuvant treatment.

**DISCUSSION**

This study has demonstrated that at least two kinds of T-kininogens (TI and TII) are present in plasma from adjuvant-treated rats. The isolation of TI- and TII-kininogens was performed by essentially the same method as for T-kininogen from non-inflamed rat plasma (9). The complete separation of TI- and TII-kininogens was achieved by a second DEAE-Sephadex A-50 step. The yield of T-kininogens from inflamed rat plasma was very high, compared with T-kininogen from non-inflamed rat plasma. Total amounts of T-kininogens before the last step were calculated to be 64 absorbance units
that there exist three kinds of T-kininogens, TII-kininogen and two kinds of TI-kininogen, in plasma from adjuvant-treated rats. Therefore, we designate the two variants of TII-kininogens as TIIa- and TIIb-kininogens, which correspond to two kinds of T-kininogens from non-inflamed rat plasma (9).

The predicted amino acid sequence for MAP was recently reported by two groups of investigation (5, 6). We call them sequence MAP-1 and MAP-2, respectively. When we compare the NH2-terminal and COOH-terminal parts of the sequences of MAP with those of TI- and TII-kininogens from plasma of adjuvant-treated rats, MAP-1 corresponds to TII-kininogen (Fig. 12B). However, MAP-2 does not completely correspond to either TI- or TII-kininogen. It is of interest that cysteine 10 in the NH2-terminal part is replaced with arginine in MAP-2.

Furuto-Kato et al. (2) and Kitagawa et al. (23) recently determined three types of cloned cDNA sequences for rat low M, kininogens, one of which, their K-kininogen, corresponds to low M, kininogen B (9) and the other two to T-kininogens judging from the sequences around the kinin moiety. We compared the predicted sequences for TI- and TII-kininogens with those for TI- and TII-kininogens from adjuvant-treated rats. The sequence of TII-kininogen reported in this paper is exactly the same as the predicted sequence from the cDNA for TII-kininogen. On the other hand, the predicted amino acid sequence of TI-kininogen is the same as that of one of the T-kininogens, TIIα-kininogen, isolated by us in this work. The mRNA for another TI-kininogen, TIIβ-kininogen, which corresponds to MAP-1, was not isolated by them. Kageyama et al. (3) reported that the mRNA levels for both T-kininogens in rat liver is increased on induction of acute inflammation by administration of lipopolysaccharide, but not for the K-kininogens (high M, kininogen and low M, kininogen B). They have also shown that mRNAs for TI- and TII-kininogens are present in equal amounts in non-inflamed rat liver (3, 23). In the previous paper (9), we demonstrated that T-kininogen from plasma of non-inflamed rats consists mainly of TI-kininogen. As described in this study, two distinct peaks of kininogens are reproducibly detected together with high M, kininogen on DEAE-Sephadex A-50 chromatography of plasma from inflamed rats, whereas only one peak of kininogen (except high M, kininogen) is detected on chromatography of plasma from non-inflamed rats. The difference in position of high M, kininogen in the two elutions may be due to differences in the volume of plasma applied, as we have
experienced this effect often on chromatography of bovine plasma. The two kininogens, T-I and T-II kininogens, showed different mobility on disc PAGE. Immuno blotting analysis of plasmas from inflamed and non-inflamed rats unequivocally demonstrated that T-II-kininogen is generated after adjuvant treatment of rats. If we assume that this result is not due to differences in the strains of rats used, T-II-kininogen synthesized in microsome may not be secreted or metabolized very rapidly after its secretion in non-inflamed rats. It is also probable that T-II-kininogen is not glycosylated in the non-inflamed rat, but glycosylated and secreted on induction of inflammation; it is also possible that T-II-kininogen may not be in plasma per se, but resident elsewhere in the non-inflamed rat. In any event, the physiological significance of T-I and T-II-kininogens in the inflammatory process remains to be established.

All the data presented by many groups of investigators, including ourselves, demonstrate that the T-kininogens are a family of kininogens which do not release kinin by the action of kallikreins and are identical or similar to MAP and a-cysteine proteinase inhibitor (2-7, 9, 19). However, their amino acid sequences are not exactly consistent. These discrepancies may be due to species differences in the rats used; and this awaits further investigation. This study has also demonstrated that T-kininogens are heterogenous in carbohydrate content. The sialic acid content of T-II-kininogen is larger than that of T-I-kininogen. Although only glucosamine was detected on analysis of the T-I and T-II-kininogens, analysis of the kinin-containing fragments demonstrated that they contain galactosamine linked to threonine 409. Heterogeneity may be also caused by differences in the amino acid residues to which carbohydrate is attached, as shown in bovine high M, kininogen (20). It has been shown that the ratio of synthesis of several plasma glycoproteins is significantly increased after induction of inflammation, together with changes in the type of oligosaccharides in these proteins, as described by Hatton et al. (21). The significance of these heterogeneities in acute phase inflammation also awaits further investigation.

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against rabbit IgG (diluted one-sixth) and developed using 4-chloro-1-naphthol as a substrate for horse radish peroxidase.

Reduction and Alkylation—After reduction with 2-mercaptoethanol, kininogen was S-pyridylethylated.

Enzymatic Treatment of Kininogen—Kininogen was incubated with pyroglutamate aminopeptidase (Bohringer Mannhein) at 37°C for 8 h and with lysyl endopeptidase (Wako Chemical Co., Osaka) at 26°C for 2 days. Digestion was performed with a molar ratio of enzyme to substrate of 1:20 in 200 ml of 0.05 M Tris-Cl, pH 9.0, containing 1.6 M urea for 60°C. Digestion of the digest from Tl-kininogen was performed with a molar ratio of enzyme to substrate of 1:20 in 200 ml of 0.05 M phosphate buffer, pH 6.5, containing 1 mM DPP. with 10 ml of sialidase (Bohringer Mannhein) and incubating for 12 h at 37°C. Sialic acid was determined according to the method of Skwa and Moore.

Fig. 3. Gel-filtration of T-kininogens on a column of Sephadex G-150. The breakthrough fractions from Tl- and TII-kininogen fraction on Sephadex G-150 column chromatography were lyophilized. The lyophilized peptide was applied to a column (4.6 x 14 cm) of Sephadex G-150, equilibrated with 0.2 M Tris-Cl, pH 8.5, containing 8.1 M urea, each 15 ml fraction was collected. (H) Tl-kininogen fraction, (M) TII-kininogen fraction. Absorbance at 280 nm. — kinin liberated by snake venom kininogenase.

Fig. 4. Hydrophobic chromatography of Tl-kininogen on phenyl 5PW. Twenty ml of Tl-kininogen fraction from the hydrophobic column, ammonium sulfate was slowly added to give a final concentration of 1.8 M with stirring. Twenty ml of the solution was applied to a column (7.5 x 75 mm) of Phenyl 5PW, equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 1.8 M ammonium sulfate. Protein was eluted with a linear gradient of ammonium sulfate from 1.06 M to 0.36 M using a PPH system. --- concentration of ammonium sulfate.

Fig. 5. HPLC of kinin-containing peptides derived from Tl- and TII-kininogens. Each of the kinase-containing peptides from Tl-kininogen (25 nmol) and from TII-kininogen (27 nmol) was incubated with lysyl endopeptidase. The digest was applied to a column (10 x 250 mm) of Cosnosil 5C18, equilibrated with 0.05 M Tris-HCl, pH 9.0, and developed using 4-chloro-1-naphthol as a substrate for horse radish peroxidase. The peptide peak which liberated kinin was cross-hatched.