THE LOCATION AND NATURE OF THE LINKAGE OF KININ MOIETY IN BOVINE KININOGEN-II

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Kininogen is a precursor protein, from which bradykinin or kallidin is released by kinin-releasing enzymes. Purifications of the kininogen have been made from bovine (Habermann, 1963, Greenbaum and Hosoda, 1963, Suzuki et al., 1965) and human plasmas (Pierce and Webster, 1966) and rabbit serum (Paskina and Yegorova, 1966). Two types of kininogen, I and II, have been found in bovine plasma (Yano et al., 1967a), and the latter with molecular weight of 49,500 has been highly purified. The investigations on chemical and physicochemical properties (Nagasawa et al., 1966, Kato et al., 1967a, b) revealed that bovine kininogen-II is an acidic glycoprotein bridged with six or seven disulfide bonds and that kinin moiety locates at the inner part of kininogen-II molecule. This paper describes the evidence that kinin moiety links in peptide bond at the central part of kininogen-II molecule.

MATERIALS AND METHODS

The purified bovine kininogen-II (Yano et al., 1967b) and S-carboxymethylated-(RCM-)kininogen-II (Kato et al., 1967a) were prepared as described previously. Commercial hog pancreatic kallikrein (EC. 3.4.4.21) was purified to the preparation free from proteolytic enzymes by DEAE-cellulose column chromatography and isoelectric fractionation with carrier amopholytes (to be publish-

ed). The bradykinin releasing enzyme purified (Sato et al., 1965) from the venom of Agkistrodon halys blomhoffii was highly purified by isoelectric fractionation with carrier amopholytes. A kinin-yielding peptide (BrCN-fragment) obtained by the method of Gross and Witkop (Gross and Witkop, 1962) from the reaction mixture of 130 mg of purified kininogen-II and 141 mg of cyanogen bromide was purified on a column (3 x 100 cm) of Sephadex G-75, equilibrated with 0.2 N acetic acid, and was purified by CM-cellulose column chromatography. N-terminal analysis was made by the dansyl method (Gray and Hartley, 1963) with 1-dimethyl-amino-naphthalene-5-sulfonyl chloride. Amino acid analysis was performed using an autoanalyser, Model JLC-3BC, Japan Electron Optics Lab. LTD., according to the method of Spackmann et al. (1958). Neutral sugar was assayed by oricinol-reaction according to the method of Fernell and King (1953).

RESULTS AND DISCUSSION

In 2 ml of 0.05 M triethylamine-CO_2 buffer, pH 8.6, 1.4 mg of kininogen-II was dissolved and incubated, respectively, with 42 μg of the purified kallikrein or 53 μg of the venom enzyme for one hour at 37°C. To the mixture 2 ml of acetone and 0.2 ml of 0.5 % dansyl chloride solution was added. After allowed to stand for 12 hours, dansylated peptide was subjected to thin layer chromatography. As shown in Fig. 1, snake venom enzyme and hog pancreatic kallikrein liberated bradykinin and kallidin respectively from native kininogen-II. Hog pancreatic kallikrein liberated trace amount of other peptide.

Fifty mg of native kininogen-II was incubated with venom brady kinin releasing enzyme at 37°C for 2 hours. The reaction mixture was applied to a column of Sephadex G-25 and the liberated kinin

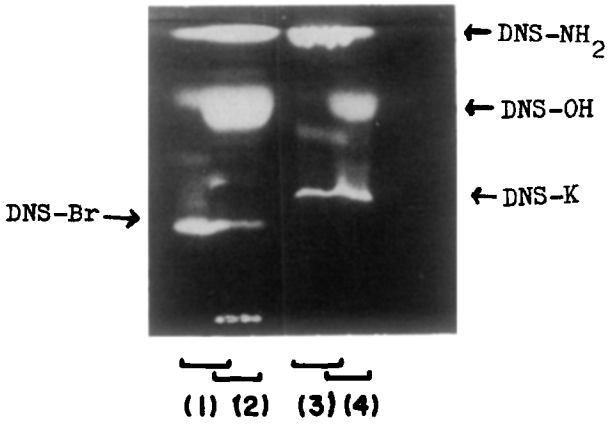

Fig. 1. Identification of dansyl-kinins by thin layer chromato graphy. Native kininogen-II was incubated with hog pancreatic kallikrein or snake venom bradykinin releasing enzyme and the liberated peptides were dansylated. Dansyl-peptides were compared with synthetic dansyl-kallidin (DNS-K) or synthetic dansyl-bradykinin (DNS-Br) by overlapping method on thin layer plates. Solvent; 2-isopropanal : methylacetate : 28% NH_3 = 35 : 45 : 20.
(1) Synthetic DNS-Br (2) Native kininogen-II + snake venom enzyme (3) Native kininogen-II + hog pancreatic kallikrein (4) Synthetic DNS-K
$DNS-NH_2$; 1-dimethyl-amino-naphthalene-5-sulfonylamide, DNS-OH; 1-dimethyl-amino-5-naphthalene sulfonic acid.

was removed from the remaining protein moiety, namely kinin-free-kininogen-II. A single protein peak was observed when the kinin-free-kininogen-II was gel-filtered through a column (2 x 90 cm) of Sephadex G-150. The sedimentation constant, $S^{0.49\%}_{20,\,w}$, of the kinin-free-kininogen-II was 3.3 S and this value was the same as that of native kininogen-II. The same results as described above were also obtained when pancreatic kallikrein was used as kinin releasing enzyme.

The disulfide bonds of kinin-free-kininogen-II (30 mg) were

reduced and carboxymethylated, and the resulting material was subjected to ultracentrifugation and gel-filtration. In the ultracentrifugation a slow moving main peak with 1.32 S was found, however, it was partially separated into two components in gel filtration on a column (2 x 70 cm) of Sephadex G-150 (Fig. 2). Neutral sugar was unequally distributed in these two components. These results indicate that two large fragments with slightly different molecular size have different sugar content. And it is strongly suggested that kinin moiety locates at approximately the central part of the polypeptide chain bridged by disulfide linkage in the kininogen-II molecule. A minoar peak with 6 S, which was observed in the ultracentrifugal pattern and in the void volume fraction on gel filtration of Sephadex G-150 seems to be an aggregate of the carboxymethylated protein.

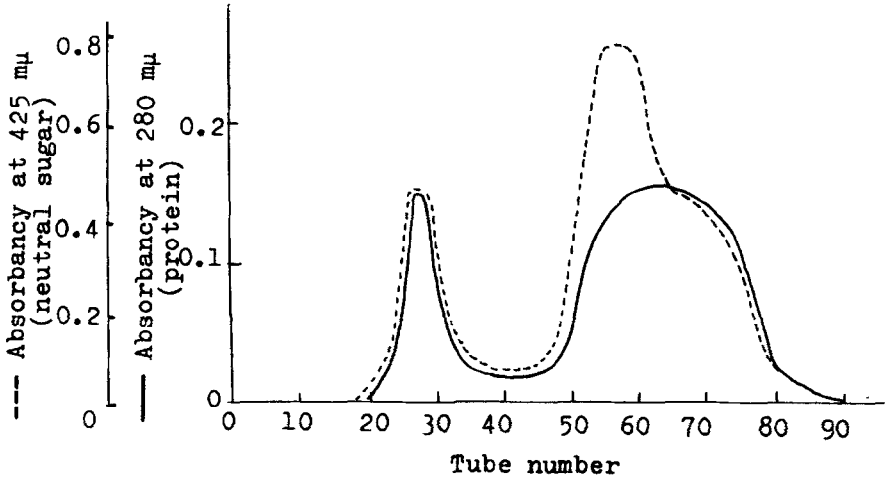

Fig. 2. Gel-filtration of S-carboxymethylated kinin-free-kininogen-II on a column of Sephadex G-150. To a column of Sephadex G-150 (2 x 70 cm), equilibrated with phosphate buffer, μ=0.1, pH 7.0, 28 mg of S-carboxymethylated kinin-free-kininogen-II was applied and eluted with the equilibration buffer. Each 3 ml fraction was collected at 4°C. From each tube, 0.5 ml of aliquots were subjected to neutral sugar analysis using orcinol reagent.

The BrCN-fragment consisted of kallidin unit and five additional amino acid residues as follows; Ser_1, Glu_1, Val_2, $Homoserine_1$. The N-terminal amino acid of the peptide was found to be lysine by dansyl and leucine aminopeptidase methods. After hydrolysis of the peptide with venom bradykinin releasing enzyme, two peptides, one basic and the other neutral, were separated by a high voltage paper electrophoresis at pH 3.5. The basic peptide was identified as kallidin from the results of amino acid analysis, paper electrophoresis and dansyl method. The N-terminal amino acid of the neutral peptide was found to be serine. The neutral peptide was digested with carboxypeptidase A and leucine aminopeptidase and the liberated amino acids were determined quantitatively by autoanalyzer. From the sequence of the liberated amino acids, the structure of the neutral peptide was determined to be Ser-Val-Gln Val-Homoser. Therefore, the whole structure of the BrCN fragment was deduced as follows, and this structure has also been confirmed by Habermann (Habermann, 1967) with the use of the Edman's degradation method.

Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Val-Gln-Val-Homoser.

It has usually been considered that the kinin moiety in kininogen-II is linked with two peptide bonds. However, the question that the C-terminal amino acid, arginine, of kinin may link to serine residue with ester bond remains (Pierce, 1968), because kinin releasing enzymes split only N-substituted arginine esters when the synthetic amino acid derivatives are used as substrates. When we examined the N-terminal amino acid of BrCN fragment by dansyl method, dansyl lysine was only detected. If the C-terminal arginine residue of the kinin links to serine by ester bond, dansyl

serine must be detected together with dansyl lysine. Therefore, the possibility that the C-terminal amino acid residue of kinin links to serine residue with ester bond was excluded completely.

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