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Further characterization of human plasma kininogens

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The substrates in plasma from which the vasoactive kinin peptides are liberated by various enzymes are known as kininogens. The literature on this subject was reviewed recently by Pierce [1] and Habermann [2]. Some investigators had isolated one, others two kininogens. Since the publication of these reviews, papers have been published in which the isolation and characterization of only one kininogen was reported [3–6]. In other publications, the isolation of two kininogens was described [7–11].

Recently, we reported in detail on the isolation of two physico-chemically and functionally different kininogens [12]. Material obtained essentially in this manner was used for the further characterization of the two substrates. Preliminary data were presented recently [13].

Isolation of kininogens. Starting material was 6 liters of ACD plasma. The kininogens obtained were purified, as described in detail before [12]. In more recent experiments, a lysine-Sepharose step was interposed early in the purification of low molecular weight (LMW)-kininogen to eliminate traces of plasminogen/plasmin, responsible for some of the loss of this substrate. Residual inhibitors (α_1-antitrypsin and α_2-antithrombin) were eliminated from the LMW-kininogen by passage through a column of Concanavalin A. In this process, over 80 per cent of the kininogen was recovered in the excluded peak and the proteinase inhibitors were retained.

With preparations isolated by the previously described procedures [12] 1 mg LMW-kininogen yielded approximately 10 μg bradykinin equivalents, when incubated with trypsin at 37° for 5 min. Approximately 8 μg kinin was released from 1 mg high molecular weight (HMW)-kininogen. Under these conditions, there is a considerable loss of intact kininogen, due to "spontaneous" activation [12]. By interposing the lysine-Sepharose step, the yield of kinin from 1 mg LMW-kininogen was raised to 16 μg bradykinin equivalents. Whether partially purified kininogens [9] or kininogens which were homogeneous by disc gel electrophoresis [12] were used, the results were essentially the same.

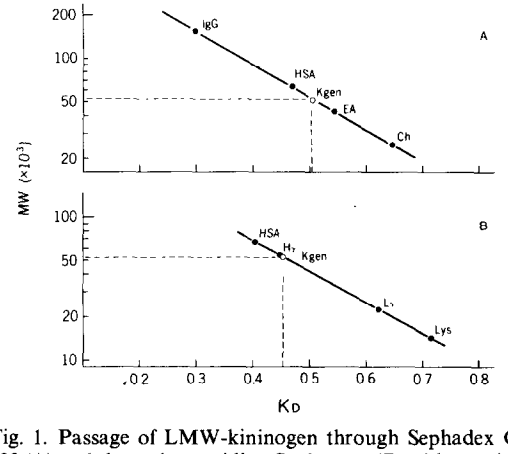

Fig. 1. Passage of LMW-kininogen through Sephadex G-200 (A) and through guanidine-Sepharose 4B, with or without reduction and alkylation (B). The Sephadex G-200 column (1·6 × 92 cm) was packed with Sephadex G-200 fine, equilibrated with 0·1 M Tris-HCl (pH 8·0) containing 0·5 M NaCl and 0·003 M EDTA. The Sepharose 4B column (1·6 × 88 cm) was equilibrated with 0·001 M Na acetate buffer (pH 4·2), containing 5·0 M guanidine-HCl. The sample applied was 1·0 ml and the flow rate averaged 4·0 ml/hr. For designation of markers, see Fig. 2.

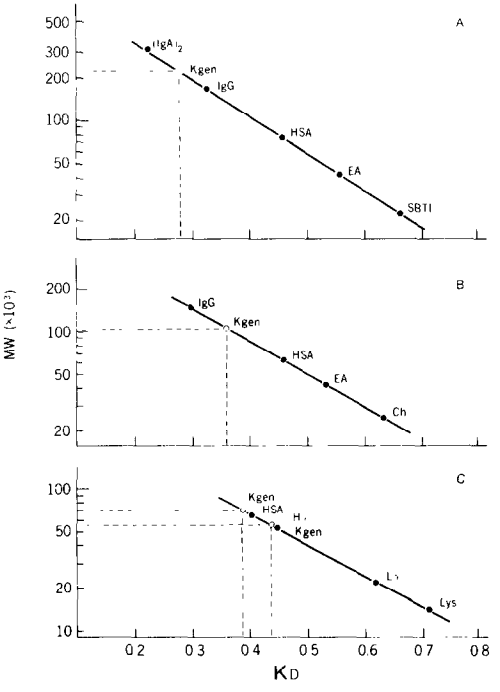

Fig. 2. Gel filtration of HMW-kininogen through Sephadex G-200 (A), guanidine-Sepharose 4B (B) and guanidine-Sepharose 4B after reduction and alkylation (C). Conditions were identical to those in Fig. 1. The following markers were used: $(IgA)_2$ = IgA dimer; IgG, HSA = human serum albumin; EA = egg albumin; SBTI = soy bean trypsin inhibitor; Ch = chymotrypsinogen; Hγ = heavy chain of IgG; Lγ = light chain of IgG; and Lys = lysozyme. K_D was calculated as described in [14].

Characterization of kininogens by gel filtration. The kininogens were subjected to gel filtration as described in Fig. 1. Prior to gel filtration in guanidine-HCl, the samples were dialyzed for 24 hr against 5·0 M guanidine, pH 4·2. In some experiments, samples were reduced in 5·0 M guanidine-HCl, 0·2 M Tris-HCl, 0·05 M dithiothreitol, pH 8·0, for 2 hr at room temperature. The reduced sample was alkylated by addition of iodoacetamide to a final concentration of 0·12 M for 45 min at 7° and the sample subsequently applied to the column.

Five runs were performed with the LMW-kininogen, both through Sephadex G-200 in Tris-HCl–NaCl and Sepharose 4B in guanidine-HCl. Six experiments were carried out with the HMW-kininogen.

Gel filtration columns were calibrated with markers of known molecular weight as shown in Figs. 1 and 2. Radioactive markers were co-chromatographed with the kininogens. The calibration curves were constructed from data obtained by multiple runs of individual markers and runs performed with mixtures of standards which were widely separated from each other by virtue of their molecular size differences or by virtue of different methods of detection such as optical density, or scintillation counting for ^{131}I or ^{125}I. The void volume (V_o) was determined

with blue dextran and the total column volume (V_t) was determined with DNP-glycine.

In the case of gel filtration in guanidine, the molecular weights of the markers were the best values obtained from the literature [14]. In the case of gel filtration in non-denaturing conditions on Sephadex G-200, the molecular weights used were those given by Andrews [15] which "best fit."

The elution of kininogens was monitored by measurements of kinin generation. The kininogen fractions obtained by gel filtration were dialyzed for 48 hr against 0·02 M phosphate-buffered saline (pH 7·4) and incubated with trypsin or plasma kallikrein. One-eighth ml kininogen was incubated with 20 μg in 0·1 ml trypsin (TPCK, 200 U/ml, Worthington, Freehold, N.J.) for 5 min at 37° and the trypsin neutralized with 24 μg in 0·1 ml lima bean trypsin inhibitor (Type II L, Sigma, St. Louis, Mo.). The kallikrein was prepared as described before [12]. Eighteen μg kallikrein (BEAe hydrolyzing activity: 380 nmoles/min/ml) was incubated for 10 min with 0·8 ml kininogen and 0·1 ml buffered saline, followed by boiling for 10 min. The cleavage product was assayed on the estrous rat uterus, standardized with synthetic bradykinin, as described elsewhere [12].

Antibody against the kininogens was prepared in rabbits. The IgG fraction of the antiserum fractionated on QAE-Sephadex [12] was isolated, concentrated, heated to 56° for 1 hr and treated with DFP (10^{-3} M final concn) to inactivate kallikrein. Normal IgG was prepared in the same manner.

Dialysis of the kininogens for 48 hr against 5·0 M guanidine-HCl, with or without reduction and alkylation, did not change the yield of kinin generated by trypsin.

When tested with trypsin, the HMW-kininogen eluting from Sephadex G-200 had an estimated molecular weight of 210,000 ± 10,000, whereas the molecular weight of the LMW-kininogen was 52,000 ± 3,000. After passage through guanidine-Sepharose 4B, the HMW-kininogen eluted with an elution volume, indicating a molecular weight of 108,000 ± 10,000. Under the same conditions, the LMW-kininogen had an approximate molecular weight of 52,000 ± 3,000. After reduction, alkylation and passage through guanidine-Sepharose 4B, the LMW-kininogen eluted with a volume equivalent to, or slightly larger than, that of the heavy chain of IgG, i.e. it remained relatively unchanged (Fig. 1). On the other hand, reduction and alkylation of the HMW-kininogen gave rise to two peaks of activity. The elution volume of the first peak corresponded to a molecular weight of approximately 70,000 ± 5,000, whereas that of the second peak corresponded to a molecular weight of 55,000 ± 5,000 (Fig. 2). When the ascending portion of the first peak and the descending limb of the second peak were concentrated and rechromatographed, the molecular weights were again 70,000 and 55,000 respectively (Fig. 3).

Recovery of kininogens after chromatography ranged between 80 and 85 per cent.

LMW- or HMW-kininogen was incubated with antibody for 1 hr at 37° followed by an incubation for 24 hr at 4°. The small amount of precipitate was spun out and the supernatant tested on the rat uterus after incubation with trypsin as described earlier. Incubation with antibody against LMW-kininogen prevented kinin generation from LMW- as well as HMW-kininogen. Similarly, antibody directed against the HMW-kininogen neutralized both substrates. Normal rabbit immunoglobulin G, prepared by the same procedure, had no effect on either kininogen (Table 1).

We have previously reported the isolation of two functionally different kininogens from human plasma [12]. The two kininogens were equally susceptible to cleavage by trypsin but differed in their cleavage by plasma kallikrein. This study has confirmed our initial observations that the two kininogens differed in molecular weight, one having

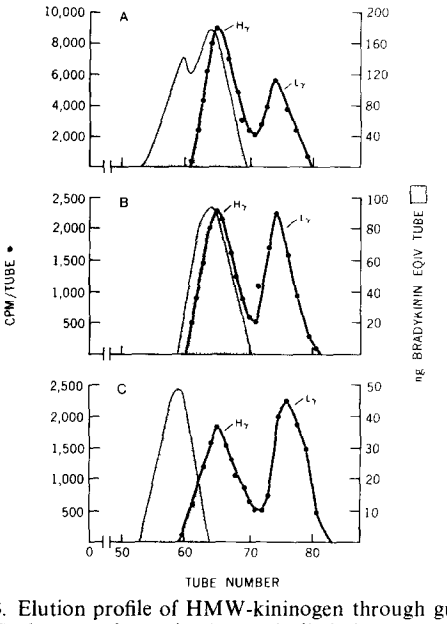

Fig. 3. Elution profile of HMW-kininogen through guanidine-Sepharose, after reduction and alkylation (A). B and C represent the rechromatography of the descending and ascending portions of the peaks shown in A. Conditions are as in Fig. 1. Approximately, 2·0 ml tube was collected and the weight of each tube determined, as suggested in [14]. Radiolabeled IgG was reduced and alkylated together with the kininogen and the elution profile (cpm/tube) of the heavy (Hγ) and light (Lγ) chains was monitored.

an apparent molecular weight of ~210,000 daltons and the other having a molecular weight of ~50,000 daltons, when examined by gel filtration in a non-dissociating solvent. Gel filtration in a dissociating solvent, 5·0 M guanidine-HCl [14], demonstrated that the HMW-kininogen had approximately twice the molecular weight of the LMW-kininogen. Reduction and alkylation of the HMW-kininogen yielded two fragments, having molecular weights of approximately 70,000 and 55,000 respectively. In con-

Table 1. Effect of anti-kininogen antibodies on low molecular weight- and high molecular weight-kininogens*

Immunoglobulin fraction added to kininogens	% Neutralization of kininogen	
	HMW-kininogen	LMW-kininogen
Anti-HMW (1·6 ml)	60	54
Anti-HMW (3·2 ml)	87	79
Anti-LMW (1·6 ml)	73	75
Anti-LMW (3·2 ml)	90	85
Normal (3·2 ml)	0	0

* Immunoglobulins were obtained by QAE-Sephadex chromatography, eluting with the excluded peak. The IgG fraction was heated to 61° for 1 hr, treated with DFP (10^{-3} M final concn), and dialyzed against phosphate-buffered saline, pH 7·4, for 24 hr. Five μg HMW-kininogen was incubated with IgG fractions having a concentration of 2·0 mg/ml. The total volume was adjusted to 3·6 ml with phosphate-buffered saline, incubated at 37° for 1 hr and then kept at 4° for 24 hr. The sample was divided into two aliquots (1·8 ml). One was boiled for 2 min in order to test the development of spontaneous kinin. The second aliquot was treated with 100 μg trypsin for 10 min, neutralized with 120 μg lima bean trypsin inhibitor, boiled for 10 min and assayed for the presence of generated kinin.

trast, the LMW-kininogen behaved as a ~52,000 dalton protein in both non-dissociating and dissociating conditions.

Earlier, we reported an apparent immunologic identity between the two kininogens, using immunodiffusion [9]. Similar observations were made by Komiya *et al.* [8] with bovine kininogens. As reported in this paper, when tested functionally (kinin liberation) antibody against one kininogen can neutralize both kininogens.

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Binding of vinblastine *in vitro* to ribosomes of Sarcoma 180 cells*

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Among the many effects of vinblastine (VLB) on cell growth is the induction of helical arrays of ribosomes [1, 2]. The association of such ribosomal complexes with fine, granular, electron-dense material has been reported [2, 3]. On the basis of its faint linearity and the presence of occasional tubular cross sections, it has been suggested that this material consists of newly formed microtubule precursor protein organized into crystals by VLB during its synthesis on the adjacent polysomes. Other work indicates, however, that VLB also may act directly upon ribosomes in the induction of such complexes. Polyribosome arrays appear in *Escherichia coli*, which is considered to contain no microtubules [1]. VLB has been shown to precipitate 65 per cent of chick oviduct ribosomes *in vitro* when added at a high concentration (3×10^{-3} M) [4]. Furthermore, Stebbings [5] has noted a considerable reduction in the numbers of ribosomes in oocytes treated with VLB that was not attributable to inhibition of RNA synthesis. Finally, various inhibitors of protein biosynthesis failed to prevent completely the formation of ribosomal aggregates [3]. The present study finds that VLB binds ribosomes *in vitro*, and attempts to assay this binding for its effect upon ribosome function.

Sarcoma 180 (S180) cells in culture (Fisher's medium with 10% horse serum) were grown in the presence of 10 μCi [^{14}C]uridine (407 mCi/m-mole) for 24 hr to label ribosomes. These cells (100×10^6) were then collected in log phase by centrifugation, washed once in saline and resuspended in 2 ml of LS buffer [10 mM KCl, 20 mM Tris–HCl (pH 7·5), 1·5 mM $MgCl_2$, 1·5 mM β-mercaptoethanol, 10 μg/ml PVS] where they were allowed to swell for 10 min at room temperature; this cell suspension was then homogenized in a Potter homogenizer. In this and in other experiments, all further procedures were performed at 0°. Unbroken cells, nuclei and mitochondria were sedimented by centrifugation at 10,000 rev/min for 6 min, sodium deoxycholate was added (0·5%) and the postmitochondrial supernatant centrifuged at 80,000 *g* for 130 min to sediment the ribosomes as a near-transparent aggregate. The ribosome pellet was rinsed three times with LS buffer to remove traces of detergent, resuspended in buffer, centrifuged again at 80,000 *g* for 130 min and resuspended in 0·20 ml of LS buffer. Subunits were prepared from the ribosome pellet isolated as above by washing with HS buffer [880 mM KCl, 50 mM Tris–HCl (pH 7·5), 12·5 mM $MgCl_2$,

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