IDENTIFICATION IN HUMAN PLASMA OF LOW M_r PROTEIN FRAGMENTS WITH ANTIGENIC DETERMINANTS OF KININOGEN

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1. Introduction

Low M_r fragments (8000–20 000) derived by kallikrein digestion of human HM_r (120 000) kininogen have long been sought for [1–5]. In [2] purified HM_r kininogen was incubated with human plasma kallikrein producing an 8000 M_r fragment isolated on Sephadex G-50. A glycopeptide (M_r 4600) and a histidine-rich peptide (M_r 8000) obtained with bovine plasma kallikrein from purified bovine HM_r kininogen have been clearly established by sequence analyses [6].

We have developed a radioimmunoassay for the detection of molecular forms of human plasma kininogen separated by SDS–PAGE [7]. It could be shown that HM_r and LM_r (58 000) kininogens share antigenic determinants in the heavy chain. Here, we show that a LM_r fragment (~8000) with the similar antigenic response is found in human plasma in the absence of activated kallikrein. After isolation 2 or 3 antigenic fragments (M_r 8000–11 000) were identified. Further these results exclude that the fragments are derivatives of the HM_r kininogen light chain but strongly suggest that they may be parts of the heavy chain. As shown in [7,8] the antigenic determinants of a kininogen isolated from Cohn's fraction IV (H_C-antigen) appear to be resistant to activated intrinsic proteinases. Attempts have been made to correlate the formation of the LM_r antigenic fragments with the intrinsic spontaneously or glass-activated kallikrein as measured on the synthetic substrate H–D-Pro–Phe–Arg–pNA [9].

Abbreviations: DFP, diisopropylfluorophosphate; EACA, ε-aminocaproic acid; EDTA, ethylenediamine tetraacetic acid; HM_r, high molecular mass kininogen; LM_r, low molecular mass kininogen; PBR, hexadimethrine bromide; PMSF, phenylmethane sulfonyl fluoride; RIA, radioimmunoassay; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

2. Materials and methods

2.1. *Plasma materials*

Single donor plasma was taken into polypropylene tubes containing 1 ml ACD anticoagulant solution (citric acid, trisodium citrate, dextrose)/9 ml blood and collected after centrifugation.

Plasma from 2 donors and human blood bank plasma (anticoagulant citrate, dextrose, phosphate, CPD) were obtained from the Finnish Red Cross Blood Transfusion Service (by courtesy of Dr G. Myllylä).

2.2. *Isolation of fragments*

QAE-Sephadex A-50 was equilibrated at ionic strength = 0.12 with 0.02 M Na-phosphate buffer (pH 8.0), 0.1 M EACA, 0.03 M EDTA, 3 mM PMSF (Sigma) or 0.1 M Tris–HCl (pH 8.0), 0.03 M EDTA, 3 mM PMSF. Normal human blood bank plasma (400 ml) was diluted 1:1.6 with buffer [2,7], poured into the siliconated QAE column (4.4 × 20 cm), prewashed with 0.04% PBR and operated at 200 ml/h, 4°C. After collecting the effluent washing was performed with 2.5 column vol. buffer. The stepwise salt elution was started by addition of NaCl to the buffer to ionic strength = 0.20. The fragment fraction was collected between ionic strength = 0.16–0.20 (av.6 runs, table 1) measured in each eluted fraction using the Philips conductivity bridge PR 9500. Repeated washing was performed with 1000 ml buffer ionic strength = 020. HM_r kininogen was collected after changing to ionic strength = 0.35 buffer.

After dialysis (until ionic strength = 0.05) the fragment fraction pool was poured into a CM-Sephadex A-50 column (4.1. × 10 cm, 100 ml/h), using 0.02 M Na-phosphate (pH 6.0) containing 0.05 M NaCl and 0.01 M EDTA and elution with a stepwise salt gradient

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0.3–0.5 M NaCl [2]. The fragment was eluted between ionic strength = 0.32–0.46.

Antigenic fragment was also isolated from a recycled QAE-fragment fraction pool using an immunoadsorbent column (0.9 × 30 cm) prepared with antiserum raised in rabbits against the kininogen H_C-antigen as in [8]. The binding capacity of the immunosorbent was 5.3 mg H_C-antigen.

2.3. *SDS–PAGE*

Analytical runs employed 5–10 μl plasma and 5–10 μg lyophilized immunoreactive fragment samples on 8% or 12% polyacrylamide (BDH Chemicals) slab gel [10] (20–25 mA at room temperature for 10 h). Identification of immunoreactive fragment was performed by slicing the gel over the entire running distance with a circular gel punch (diam. 3.7 mm) fitting the punched holes used in Ouchterlony double diffusion analysis [11]. Control runs with protein markers were run in parallel. Serva blue R was used for staining.

Preparative runs were performed on 12% slab gels (150 × 150 × 2 mm) with 13 mg total protein. The gels were run at 40 mA for 10 h, after which the immunoreactive fragment was located by Ouchterlony double diffusion analysis as described. Antigenic fragment was located and recovered between M_r 8000–11 000 from the sliced (1 × 1 × 2 mm) gel pieces by elution with 10 ml distilled water with shaking for 20 h (22°C). SDS and salt were removed on Sephadex G-25.

2.4. *Kallikrein*

Glass contact activation of plasma kallikrein was performed as in [7]. Kallikrein activity was determined using 0.09 mM synthetic substrate H–D-Pro–Phe–Arg–pNA (S-2302, Kabi). One amidase unit is equal to 1 μmol *p*-nitroaniline released/min [12].

2.5. *Antisera*

Monospecific antisera against immunologically pure LM_r-kininogen [13] and the kininogen H_C-antigen were raised in rabbits [8]. The titers were 0.3 mg/ml and 1.0 mg/ml, respectively [14].

2.6. *Quantitation of antigenic fragments*

Single radial immunodiffusion [15] was used for quantitation of LM_r kininogen fragments after ion-exchange chromatography.

2.7. *Radioimmunoassay*

Kininogen H_C-chain (10 μg) was radioiodinated to spec. act. 25 μCi/μg. Radioimmunoassay was performed using the monospecific antikininogen H_C-chain serum as in [7].

3. Results

The results obtained by RIA with eluates of gel slices after SDS–PAGE of 10 μl single donor plasma are shown in fig.1. The immunoreactive fragment is

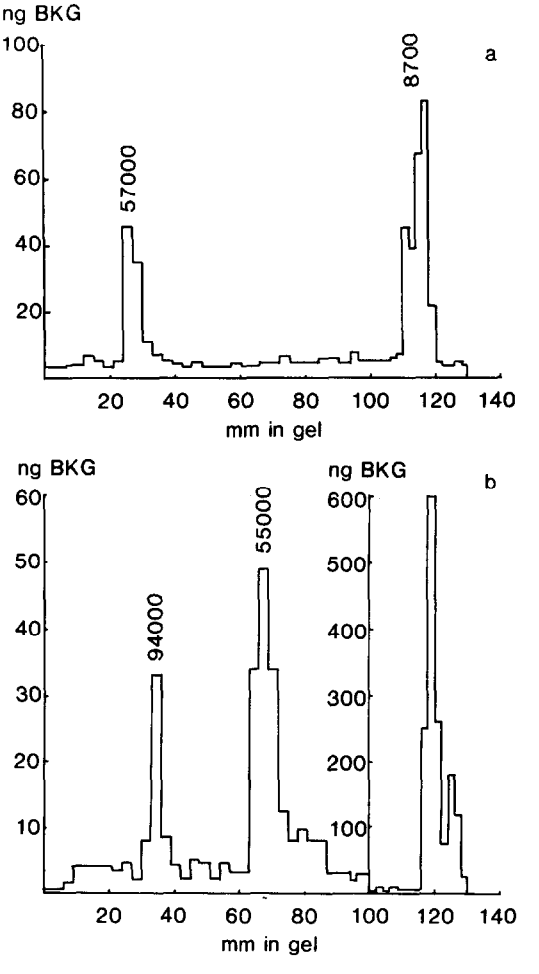

Fig.1. (a) Immunoreactive fragment, M_r 8700, from 10 μl single donor plasma together with LM_r kininogen, M_r 57 000, assayed by radioimmunoassay (ng BKG) of 50 μl eluates after separation on 12% SDS–PAGE; (b) the same analysis using 8% SDS–PAGE and 150 μl eluates showing HM_r 94 000, LM_r 55 000 and fragment moving in the front.

observed at M_r 8700 together with LM_r kininogen 57 000 (fig.1a). HM_r kininogen could not be detected with this amount of eluate but appeared at M_r 94 000 with 3 times more eluate from an 8% SDS–PAGE.

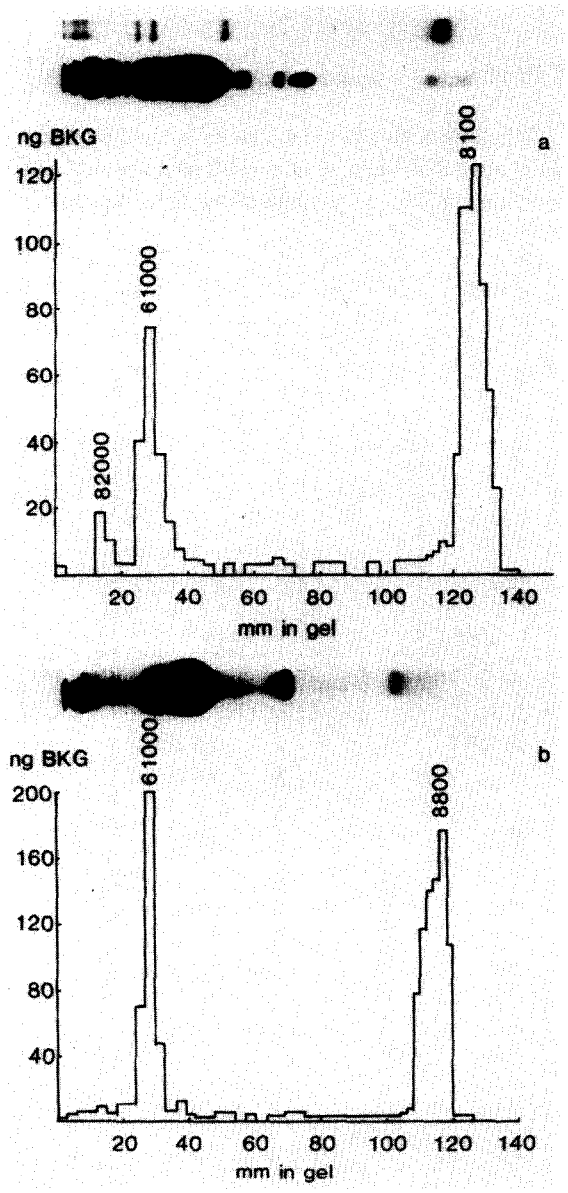

Fig.2. (a) Immunoreactive fragment, M_r 8100, from 10 μl two donors plasma together with HM_r 82 000, LM_r 61 000 assayed by radioimmunoassay (ng BKG) of 50 μl eluates after separation on 12% SDS–PAGE; (b) a similar run of recycled QAE-fragment preparation (7 μg immunoreactive kininogen) showing the fragment at M_r 8800. The respective stained gels run in parallel are shown together with molecular markers M_r 78 000, 68 000, 43 000, 12 000 (from left to right).

Fig.1b shows the fragment separately in the diagram due to the high content and uncertainty of M_r since it was found close to the marker on the gel. In a similar run with two donors plasma 3 different immunoreactive kininogens were located (fig.2a). The unusually high content of immunoreactive kininogen in this plasma (see table 1, I) acounds for the appearance of HM_r kininogen already with 50 μl eluate. These results illustrate only qualitatively the immunoreactive kininogen components. It is unknown to what extent SDS may influence the antigenic reaction. It is therefore not possible to compare quantitatively at this moment the antigenic response of the fragment, which appears to be too high compared with the other components. Furthermore, as seen in fig.2b, an M_r 61 000 component was also found in the QAE-fragment fraction pool.

Fig.3 shows the isolation of the fragment fraction by step-wise salt elution after QAE-chromatography, clearly separated from the LM_r kininogen fractions in the effluent, in which abundantly free kallikrein could be measured despite the use of inhibitor (PMSF), and from the HM_r kininogen eluted at ionic strength = 0.35. Recycling on QAE-Sephadex of the fragment pool obtained in the first run with 400 ml CPD plasma submitted to glass contact activation (table 1, III) yielded a homogeneous fraction illustrated in fig.3 (inset).

Table 1 summarizes the separation of immunoreactive kininogen by QAE-chromatography in 3 frac-

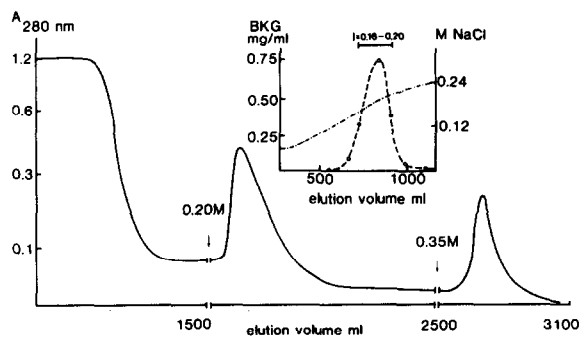

Fig.3. QAE–Sephadex A-50 chromatography of 400 ml human plasma collecting the fragment fraction by stepwise elution after ionic strength = 0.2, protein profile, A_{280} (——); for kininogen content see table 1, I. The inset figure shows the recycling on QAE–Sephadex A-50 of the fragment fraction pool in a linear salt gradient between ionic strength = 0.08–0.24 (·–·). Immunoreactive kininogen (BKG) assayed by single radial immunodiffusion (o———o).

Table 1
Immunoreactive kininogen in LM_r, fragment (M_r 8000–11 000) and HM_r fraction pools separated by stepwise salt elution after QAE-chromatography compared with kallikrein activity on H–D-Pro–Phe–Arg–pNA in the starting plasma

Prep.	Kallikrein (mg/ml)		Immunoreactive kininogen (mg)			Kininogen mg/ml plasma	
	Spontaneous	Glass-contact activated	LM_r	Fragment	HM_r	Total recovered	Starting plasma
I	0	–	128.1	24.2	15.1	0.372	0.358
II	3.9–13.4	–	101.4	5.1	15.7	0.301	0.306
III	0	4.2–5.8	80.5	21.8	9.1	0.279	0.269
IV	±	1.4–9.9	82.4	19.3	2.9	0.262	0.283
V	+	7.2	73.8	19.4	10.9	0.260	0.272
VI	±	4.9	86.2	9.3	11.8	0.268	0.286

U/ml range given for increase after dilution of the plasma

Immunoreactive kininogen determined as in [8] by single radial immunodiffusion using monospecific anti-kininogen serum against the antigen isolated from Cohn's fraction IV (H_C-chain)

I, 450 ml starting plasma from 2 donors; V, VI, chromatographed without addition of inhibitors

tion pools containing antigenic fragment, LM_r and HM_r kininogen. Autoactivated kallikrein was found in one of the plasma samples (table 1, II) despite protection against contact activation. The activities obtained after glass-contact were between 1.4–9.9 U/ml.

Purification with immunoadsorbent was performed with the dialysed and lyophilised preparation obtained from a recycled QAE-fraction pool (fig.3, inset) containing 1.8 mg immunoreactive kininogen/69 mg protein. All kininogen was retained by the column. After elution with 8 M urea 0.67 mg kininogen (37%) was recovered. This preparation was used for immunization as in [8]. As shown in fig.4a the anti-fragment serum responds antigenically to the LM_r, HM_r and H_C-antigens. This shows that the fragment shares at least one antigenic determinant with these molecules as sustained in fig.4b. This is further sustained in fig.5 showing identical RIA inhibition curves produced by the QAE-fragment fraction pool and fragment further purified from the QAE-pool by SDS–PAGE in comparison with the H_C-antigen, HM_r and LM_r kininogens.

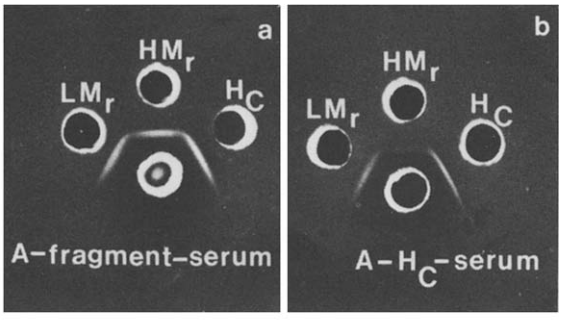

Fig.4. Characterization by double immunodiffusion of (a) the anti-H_C-chain serum and (b) the anti-fragment serum showing antigenic identity of LM_r (5 μg), HM_r (5 μg) and the H_C-chain (2.5 μg). In center wells 30 μl anti-H_C-serum and anti-fragment serum, respectively.

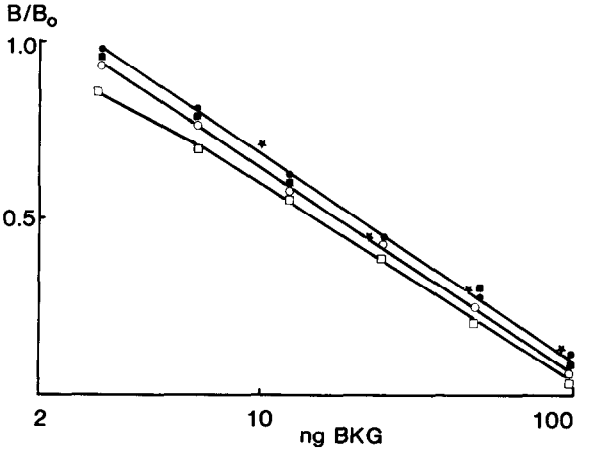

Fig.5. Radioimmunoassay inhibition curves showing antigenic identity of fragment in the QAE-fraction pool (▪) and fragment, M_r 8000–11 000, isolated from the QAE-pool by SDS–PAGE (⋆) with HM_r kininogen (□), LM_r kininogen (○) and the H_C-antigen (●). B/B_0 is the ratio of radioactivity (^{125}I-labeled H_C-antigen) bound to anti-H_C-serum in the presence and absence of inhibitor.

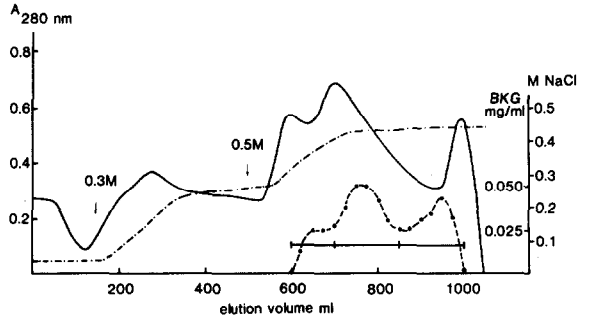

Fig.6. CM–Sephadex A-50 chromatography using stepwise elution of the fragment fraction pool from QAE-chromatography. Immunoreactive fragment(s) (BKG) assayed by single radial immunodiffusion (o———o) was eluted between ionic strength = 0.32–0.46 (·—·); the content of kininogen determined in the pools collected as indicated; protein profile, A_{280} (——).

The QAE-fragment fraction pool was submitted to CM-Sephadex chromatography shown in fig.6. Three immunoreactive fractions were obtained containing 1.5 mg, 2.2 mg and 2.1 mg (total 5.8) kininogen, respectively.

4. Discussion

There are two different approaches in isolating plasma kininogens:

(1) After blocking proteinases with DFP, which yields only HM_r 110 000–120 000 [1,2];
(2) Using blocking with other inhibitors of activated kallikrein produced during preparative procedures.

We have applied the latter method allowing activation of F XII by glass contact during preparation. By this method, allowing binding of activated proteinases to natural inhibitors in plasma and to a LM_r kallikrein inhibitor (PMSF) at least 3 HM_r molecular forms between 105 000–85 000 were earlier detected by RIA after elution from SDS–PAGE [7]. The present finding that a fragment with $M_r \sim 8000$ also is found in the fresh human plasma lacking kallikrein activity is particularly intriguing (fig.1,2) and may suggest that this fragment is a degradation product formed by other intrinsically activated proteinases connected with coagulation and fibrinolysis. The possibility that fragment may be formed from HM_r kininogen is so far unclear although some results seem to indicate this (table 1, IV). The presence of fragment in the fresh plasma may further suggest immunological cross reactivity with some other plasma protein. As shown in fig.6 several antigenically similar LM_r fragments were found. It is of interest to note (fig.4,5) that the fragment responds immunologically to the same determinant as found in LM_r, HM_r and the H_C-antigen located in the heavy chain [7,8].

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