A RADIOIMMUNOASSAY FOR THE DETECTION OF MOLECULAR FORMS OF HUMAN PLASMA KININOGEN

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1. Introduction

Kininogen is a heterogenous plasma glycoprotein and source of the vasoactive kinin peptides. Two main forms of human kininogen are well known, the high molecular mass (HM_r 120 000) and the low molecular mass (LM_r 50 000–78 000) kininogens [1–3]. HM_r kininogen functions as a cofactor in the contact activation of F XIIa and F XIa [4]. The cascade reactions leading to the formation of fibrin in the intrinsic pathway of blood coagulation also involve activation of kallikrein (EC 3.4.21.8) in an amplifying feedback mechanism between prekallikrein and F XII. A small amount of surface-bound F XII has been shown to catalyze the formation of low levels of kallikrein. Whether these reactions shown to occur in vitro when blood plasma comes into contact with glass occur under physiological conditions is not yet known [5,6].

The two molecular forms differ primarily by the structure of their respective light chain, which in the HM_r kininogen contains a histidine-rich fragment responsible for the coagulation effect and covalently bound to the heavy chain through the vasoactive peptide segment (bradykinin). However, LM_r is present in normal human plasma in a higher concentration while HM_r kininogen constitutes an average 15% of the total kininogen content [7,8]. A relationship between the two molecular forms has been suggested [7]. A common nominator appears to be the shared antigenic determinants in the heavy chains [1,9,10].

In studies so far LM_r and HM_r kininogens have been characterized by content of active kinin segment or coagulating activity. Here, we describe a radioimmunoassay method for the identification after separation on SDS–PAGE of various molecular forms of HM_r kininogen produced by activation of intrinsic kallikrein, LM_r kininogen, a kininogen from Cohn's fraction IV and the respective heavy chains.

Abbreviations: BKG, kininogen; BKG-C, immunoreactive kininogen from Cohn's plasma fraction IV; cpm, counts per minute; DFP, diisopropylfluorophosphate; HM_r, high molecular mass; LM_r, low molecular mass; PBR, hexadimethrine bromide, Aldrich-Europe; PMSF, phenylmethane sulfonyl fluoride; RIA, radioimmunoassay; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TES, *N*[tris-(hydroxy-methyl)methyl-2-amino]ethanesulfonic acid

2. Materials and methods

2.1. *Preparation of kininogen*

HM_r-BKG (~105 000–85 000 M_r by SDS–PAGE) was prepared from 400 ml normal human blood bank plasma, diluted 1:1.6, according to the procedures in [3] using 0.1 M Tris–HCl buffer (pH 8.0) containing 3 mM EDTA and PMSF (Sigma Chemical Co.) and 0.04% PBR. The 0.35 M NaCl eluted fraction pool was dialyzed, precipitated at 50% ammonium sulphate saturation and chromatographed on SP–Sephadex C-50 [2]. LM_r-BKG (~60 000 M_r) was prepared as in [8]. Immunoreactive kininogen (BKG-C, single polypeptide chain, ~60 000 M_r) was purified from freshly precipitated Cohn's plasma fraction IV [10]. The purity, amounts, homogeneity and approximate M_r-values were estimated by double diffusion, single radial immunodiffusion analyses [11–13] and SDS–PAGE [14].

2.2. *SDS–PAGE*

BKG samples were run by SDS–PAGE (8% slab gel) native and after reduction with 5% mercaptoethanol (Fluka AG) [14]. The protein was recovered from the sliced (3 mm) polyacrylamide (BDH Chemicals

Ltd.) gel strips by elution with 0.1 ml TES buffer (pH 7.35), 0.15 M NaCl [15], or with 0.2 ml 0.15 M NaCl and shaking for 18 h (22°C). Control runs with the protein samples were run in parallel and stained with Serva blue R.

2.3. *Preparation of antisera*

Monospecific antisera against the immunologically pure antigens LM_T-BKG and BKG-C were raised in rabbits [16]. The titers were 0.3 mg/ml and 1.0 mg/ml, respectively [17].

2.4. *Determination of kallikrein*

Plasma kallikrein was measured using 0.09 mM of the synthetic substrate H–D-Pro–Phe–Arg–pNA (S-2302, Kabi) in 0.1 M sodium phosphate buffer (pH 7.6) containing 0.15 M NaCl; 100 μl plasma was added to 600 μl substrate solution at 37°C and the absorbance was recorded at time intervals at 405 nm. One amidase unit is equal to 1 μmol *p*-nitroaniline released/min [18].

2.5. *Activation of plasma kallikrein*

Glass-contact activation of the plasma used for the isolation of HM_T-BKG was performed in a glass beaker left for 24 h at 4°C with gentle magnetic stirring. The activity was measured before and after dilution of the plasma and was 0.0105 U and 0.0145 U/ml plasma, respectively. In the non-glass contact plasma there was no activity on H–D-Pro–Phe–Arg–pNA. Total activation of plasma prekallikrein in plasma was performed using 90 μl of the plasma sample incubated 3 min at 37°C with 800 μl 1:10 diluted Cephotest® (Nyegaard, Oslo) in 0.1 M sodium phosphate buffer (pH 7.6), 0.15 M NaCl. Substrate was added to give the final concentration of 0.09 mM (210 μl) in the cuvette and the change of absorbance was recorded as before.

2.6. *Radiolabelling of kininogen*

Radiolabelling was performed using the chloramine T method [19] with 10 μg immunoreactive BKG dissolved in 25 μl 0.05 M sodium phosphate buffer (pH 7.5) was iodinated with 1 mCi $Na^{125}I$ (New England Nuclear, carrier free) diluted in 25 μl 0.5 M buffer. Chloramine T (Merck p.a.) 100 μg in 25 μl 0.05 M buffer was added at 0°C. After the reaction was stopped with 240 μg sodium metabisulphite (BDH) in 100 μl buffer, KI (Merck p.a.) 2 mg in 200 μl buffer was added as carrier. Radiolabelled BKG was separated from free ^{125}I by gel filtration on Sephadex G-75 (0.8 cm × 32 cm) with 0.07 M sodium diethylbarbiturate (pH 8.6). The column was equilibrated with the buffer containing 0.2% gelatine (Sigma, G-2500, from swine skin). Radioactivity was measured in a Wallac GTL 300–500 gamma sample counter. Specific activity was ~10–20 μCi/μg immunoreactive BKG. The protein was stored at −20°C 1:10 diluted in 0.05 M sodium phosphate buffer (pH 7.5) containing 0.2% gelatine, 0.15 M NaCl and 0.01% NaN_3, used in all further dilutions.

2.7. *Radioimmunoassay*

Samples of 5–100 μl SDS–PAGE gel strip eluates were analyzed by radioimmunoassay. The RIA was performed by incubating 0.2 ml samples or BKG standards, 0.1 ml radiolabelled BKG (5000–10 000 cpm) with 0.1 ml diluted antiserum calibrated to bind 50% of the maximal radioactivity at 4°C for 18 h. Normal rabbit serum 5 μl was added as carrier. Antibody bound ^{125}I-BKG was precipitated by adding 0.25 ml 1:10 diluted sheep anti-rabbit-IgG [20] and incubated

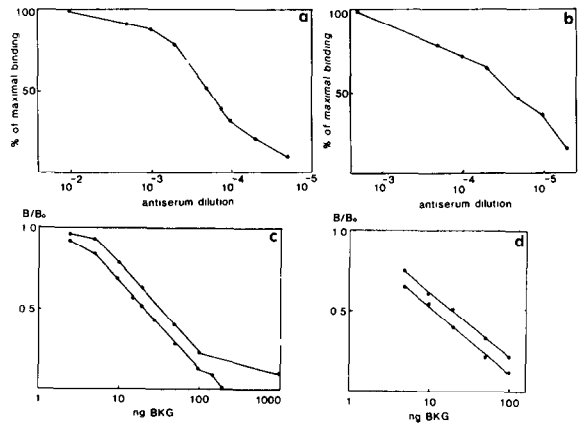

Fig.1. Antibody binding curves obtained using (a) anti-LM_T-BKG serum (titer 0.3 mg/ml) dilutions 10^{-2} to 5×10^{-4} with ^{125}I-labelled LM_T-BKG and (b) anti-BKG-C serum (titer 1 mg/ml) dilutions 5×10^{-2} to 2×10^{-5} with ^{125}I-BKG-C in the radioimmunoassay procedure without unlabelled kininogen as inhibitor. The maximal precipitation of ^{125}I-labelled kininogen using the highest antibody concentration was (a) 60% and (b) 86%; radioimmunoassay standard curves obtained using (c) LM_T-BKG for inhibition of binding of ^{125}I-LM_T-BKG (○) and BKG-C (●) for inhibition of binding of ^{125}I-labelled BKG-C to the corresponding antisera, (d) LM_T-BKG for inhibition of binding of ^{125}I-labelled LM_T-BKG to anti-LM_T-BKG serum (○) and anti-BKG-C serum (●). B/B_0 is the ratio of radioactivity bound in the presence of inhibitor and absence of inhibitor (assayed in triplicate samples).

for 2 h at 22°C with shaking. The precipitate was collected by centrifugation (5000 × g, 4°C) and counted for radioactivity in the gamma sample counter.

3. Results and discussion

The antibody binding curves for the antisera against LM_r-BKG and BKG-C are shown in fig.1a,b indicating that 50% binding was obtained at the final dilution 1:12 800 and 1:160 000, respectively. The inhibition curves of these proteins with their corresponding antisera (fig.1c) are linear from 5 ng (detection limit) to 100 ng which illustrates the measuring range applied in these studies. When both antisera were tested against LM_r-BKG parallel dose response curves were obtained (fig.1d). These two antisera were accordingly used independently in the present radioimmunoassay analyses. Recent approaches for determination of HMW and LMW kininogen by RIA report similar sensitivity of the assay [21,22].

Fig.2a demonstrates that three variants of HM_r kininogen can be shown by RIA eluted with 100 μl TES buffer yielding 27 ng, 76 ng and 70 ng for the molecular forms 105 000, 90 000 and 86 000, respectively, per 10 μl eluate. In our preparation the 120 000 M_r form of HMW-BKG was not present apparently as a consequence of degradation by intrinsic kallikrein activated by glass contact. We estimated the degree of activation to ~5% of the total kallikrein activity 0.290 ± 0.037 units/ml on H–D-Pro–Phe–Arg–pNA obtained with Cephotest®. After QAE-chromatography ~10% of the total immunoreactive kininogen (9 mg) was collected in the HM_r fraction pool, which is within the normal distribution range. Instead of DFP the PMSF inhibitor was applied in

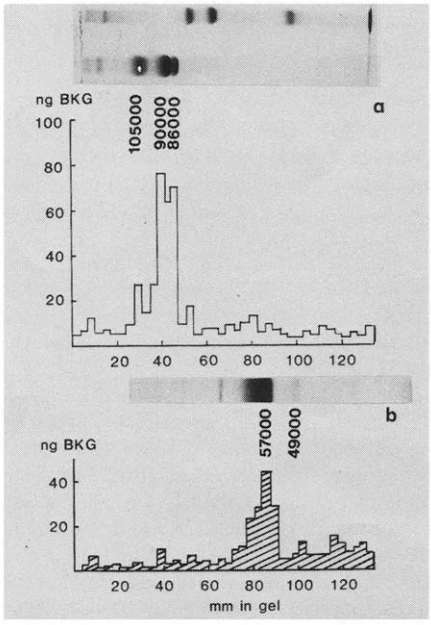

Fig.2. Radioimmunoassay of gel strips sliced and eluted after SDS–PAGE (8%) runs of molecular variants of HM_r kininogen, in (a) 15 μg immunoreactive kininogen (open) against anti-BKG-C serum, (b) 20 μg reduced (shaded) 85 000 HM_r against anti-LM_r serum. The respective stained (Serva blue®) gels run in parallel showing the position of the heavy and light chains; molecular markers (M_r): 78 000, 68 000, 43 000 and 12 000 (from left to right). The RIA was performed using the respective kininogen standards.

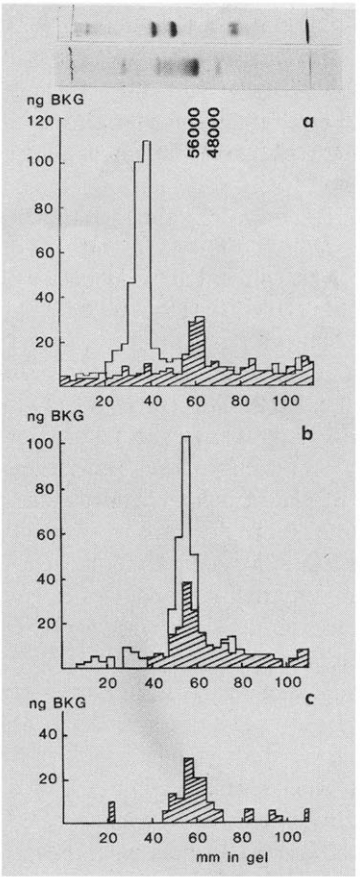

Fig.3. Radioimmunoassay of gel slices after SDS–PAGE runs of 15 μg native (open) and reduced (shaded) kininogens: (a) HM_r kininogen molecular forms (105 000–85 000 M_r), seen after reduction in the stained gel, RIA against anti-BKG-C serum with BKG-C standard; (b) LM_r kininogen native (58 000 M_r) and after reduction against anti-LM_r-kininogen serum with LM_r kininogen standard; (c) reduced BKG-C kininogen against anti-LM_r kininogen antiserum.

these studies. The degradation of HM_r was restricted by this inhibitor known to inhibit kallikrein but not the activation of prekallikrein. This agrees with [15] where degradation of purified HM_r kininogen and kallikrein in vitro and showed that 121 000 M_r kininogen was converted to an intermediate of 102 000 M_r and finally to an end product of 95 000 M_r, using SDS–PAGE and coagulation activity. As shown in fig.2,3 degradation produced still another HM_r derivative of kininogen with 85 000 M_r. Regardless of apparent enzymic cleavage these results demonstrate that intrinsic proteinase activation does not affect the antigenic determinant in the heavy chain.

As demonstrated in fig.2b with reduced HM_r kininogen using the anti-LM_r kininogen serum only the heavy chain responds in RIA. This agrees with [1] where, by immunoelectrophoresis using specifically adsorbed antisera, the light chain was suggested to contain a unique antigenic determinant that distinguishes HM_r kininogen from LM_r kininogen.

The size of the H- and L-chain obtained from these molecular HM_r forms of kininogen (fig.3a) agree with results of ~68 000–58 000 M_r and ~37 000–48 000 M_r, respectively, presented by others on SDS–PAGE with reduced 110 000–120 000 HM_r kininogen [1–3]. The RIA in fig.3a detects only the H-chain after reduction depending on the antiserum used. As shown in [9] the BKG-C antigen has the characteristics of the H-chain of LM_r and HM_r kininogen, verified in fig.3c showing only one component after reduction of BKG-C, which corresponds in size to the H-chain in fig.3a. So far there are no available data on the L-chain of human LM_r kininogen. With bovine LM_r kininogen it has been reported to have 4800 M_r [23]. The apparently same M_r-value obtained on SDS–PAGE with LM_r kininogen before and after reduction is so far unexplained (fig.3b) but may depend on the carbohydrate structure of the H-chain. As shown in [23] no carbohydrate was detectable in the bovine LM_r kininogen L-chain and there is only a slight difference in the carbohydrate content of the H-chains of bovine HM_r and LM_r kininogens [24]. Glycopeptides also routinely migrate slower than polypeptides of the same M_r-value when subjected to SDS–PAGE [25]. Further favourable evidence is the observation [26] that LM_r kininogen behaved as a 52 000 M_r protein in both non-dissociating and dissociating conditions. This is further strengthened by the considerable variation of M_r-values reported for human LM_r kininogen depending on the method [7,8,26,27].

We describe a method for the detection of molecular variants of human plasma kininogen and verify by radioimmunoassay the earlier suggestions [1,9,10] that the H-chain of HM_r and LM_r kininogens share antigenic determinants also found in the antigen isolated from Cohn's plasma fraction IV.

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