Purification of Hageman Factor (Factor XII) on Columns of Popcorn-Agarose

By Oscar D. Ratnoff, Barbara Everson, Virginia H. Donaldson and Bridget H. Mitchell

Purification of Hageman factor (HF, factor XII) from human plasma is a tedious procedure and the product is not always in the precursor form. Hohima has described a protein derived from corn kernels that inhibits the enzymatic properties of HF. This inhibitor binds to the precursor form of HF. Rapid purification of HF was achieved by using as the major purification step adsorption of this clotting factor to popcorn inhibitor bound to agarose. The product had a specific activity of 50.0 to 67.1 coagulant units of HF per milligram protein, and the yield was 33% to 40% of the HF content of the starting plasma. The purified protein displayed a single band upon unreduced or reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis and less than 0.1% was in an activated form, as measured in coagulant assays. The technique described is more rapid and reliable than methods described earlier.

We reported recently that Hageman factor (HF, factor XII) in its precursor state is readily complexed to popcorn inhibitor that has been bound to cyanogen bromide agarose, and that HF can then be eluted from this matrix. This procedure has allowed us to develop a simplified method for the purification of HF that is essentially uncontaminated with activated forms of this clotting factor. This has made it possible to purify HF more readily and more reliably than by earlier methods, and appears to avoid contamination with activated species of HF that are sometimes found in preparations separated by other techniques.2,3

MATERIALS AND METHODS

After obtaining informed consent as approved by our institutional committee on the protection of the rights of human subjects, blood for purification of HF was drawn from the antecubital veins of volunteer donors into one-fiftieth volume of 0.5 mol/L sodium citrate buffer (pH 5.0) containing 31 mg/mL of benzamidine hydrochloride (Sigma Chemical Co, St Louis, Mo) and 10 mg/mL of hexadimethrine bromide (Polybrene, Sigma). The blood was centrifuged at 2 °C for 20 minutes at 12,000 g in 250 mL polycarbonate bottles (Du Pont Co, Wilmington, Del). The plasma was withdrawn with polystyrene pipettes, and the plasma recentrifuged in the same manner. Blood from patients with Hageman trait, classic hemophilia (factor VIII “deficiency”) or parahemophilia (deficiency of procoagulant or factor V) was withdrawn into the same volume of hexadimethrine bromide (Polybrene, Sigma). The blood was anticoagulated with polystyrene pipettes, and the plasma recentrifuged without benzamidine or hexadimethrine bromide and anticoagulant in 90% (± 2%) and less than 0.1% was in an activated form, as measured in coagulant assays. The technique described is more rapid and reliable than methods described earlier.4

Popcorn-agarose HF inhibitor was prepared and, after use, regenerated as previously described.1 Other reagents used included lysine agarose,4 diethyl(2-hydroxypropyl)aminoethyl Sephadex A-50 (QAE-Sephadex, Pharmacia Fine Chemicals, Piscataway, NJ), sulfopropyl Sephadex C-50 (SP-Sephadex, Pharmacia), bovine albumin (crystallized, Pentex, Miles Laboratories, Elkhart, Ind), ammonium acetate (Sigma), ammonium sulfate (ultrapure, Schwarz-Mann, Cambridge, Mass), hexadimethrine bromide (Polybrene, Sigma, St Louis), benzamidine hydrochloride (Sigma), tissue thromboplastin (rabbit brain, Sigma, suspended in accordance with the manufacturer’s directions), soy bean phosphatides (Centrolene “P”, the gift of Central Soya Co, Fort Wayne, Ind), kaolin (acid-washed, NF, Fisher Scientific Co, Fairlawn, NJ), streptokinase (Streptase, Behringwerke AG, Marburg, W. Germany), H-D-valyl-leucyl-lysine (4-nitroanilide) hydrochloride (S2251, Helena Laboratories, Beaumont, Tex), and H-D-prolyl-L-phenylalanin-L-arginine (S2302, Helena).

The buffers used included sodium phosphate buffer (0.1 mol/L, pH 7.5), barbital-saline buffer (0.025 mol/L sodium barbital, 0.125 mol/L sodium chloride, pH 7.5), barbital-saline-albumin buffer (barbital-saline buffer containing 1% bovine albumin), barbital-saline-Polybrene buffer (barbital-saline buffer containing 100 mg/L hexadimethrine bromide), Tris buffer (0.025 mol/L Tris(hydroxymethyl)aminomethane, 0.002 mol/L benzamidine hydrochloride, 50 mg/L hexadimethrine bromide, 0.1 mmol/L disodium ethylene diamine tetraacetic acid, 0.1 mol/L sodium chloride, and enough 4N hydrochloric acid to bring the pH to 8.0), acetate buffer (0.15 mol/L sodium acetate, 0.15 mol/L sodium chloride, and enough 4N hydrochloric acid to bring the pH to 8.0), acetate buffer (0.05 mol/L sodium acetate, 0.15 mol/L sodium chloride, pH 5.2), acetate-Polybrene buffer (acetate buffer containing 100 mg/L hexadimethrine bromide), and ammonium acetate–sodium chloride buffer (0.1 mol/L ammonium acetate in 1.0 mol/L sodium chloride and 100 mg/L hexadimethrine bromide, pH 6.8). The ammonium acetate–sodium chloride buffer was prepared freshly for each experiment. All buffers used for the preparation of HF contained 0.02% sodium azide.

Except as noted, all glassware was rinsed with dichloro-octadecyltetrasiloxane (SurfSil, Pierce Chemical Co, Rockford, Ill) and dried overnight at room temperature. The Bünchert funnels used were silicon-coated in the same way. All dialyses were carried out in Spectrapor cellulose casings (mol wt cutoff 12,000 to 14,000 kD, Spectrum Medical, Los Angeles, Calif). All centrifugations were at 4 °C, those at 12,000 g in a Sorvall RC-2 centrifuge (Dupont Co, Newton, Conn), and those at 800 g in an International PR-2 centrifuge (Damon/International Equipment Co, Needham, Mass).

To purify HF, the plasma was first depleted of plasminogen by mixing at room temperature in a polycarbonate beaker approximately 300 mL of citrated plasma, prepared as described above and diluted with an equal volume of distilled water, with 150 mL settled volume of a suspension of lysine-agarose that had been equilibrated aged 21 to 40 and stored at –70 °C as described earlier.4 This pool was arbitrarily defined as containing 1.00 U/mL of each clotting factor tested.

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in 0.1 mol/L sodium phosphate buffer (pH 7.5). The mixture was stirred gently for 30 minutes and allowed to stand for an additional hour. It was then filtered through a B"{u}chner funnel fitted with Whatman No. 541 filter paper and the plasminogen-depleted plasma was collected by suction into an Erlenmeyer flask. The gel was washed with approximately 50 mL of Tris buffer, combining the filtrate with the filtered plasma. The filtrate was then dialyzed overnight at room temperature against two changes of 6 L of Tris buffer (pH 8.0).

The dialyzed plasma was transferred to a polycarbonate beaker and mixed at room temperature for one hour with 500 mL settled volume of QAE-Sephadex gel that had been equilibrated with Tris buffer. The mixture was allowed to stand at room temperature for 30 additional minutes and was then filtered by suction through a B"{u}chner funnel lined with Whatman No. 541 filter paper. The QAE-Sephadex gel, to which HF was adsorbed, was washed with 5 L of Tris buffer. The gel was transferred to a polycarbonate beaker and mixed with 150 mL of Tris buffer containing 0.5 mol/L sodium chloride. The mixture was refiltered through a B"{u}chner funnel as before, collecting the filtrate in an Erlenmeyer flask and saving the filtrate. The gel was again transferred to a polycarbonate beaker, mixed with Tris buffer containing 0.5 mol/L sodium chloride and refiltered, a process repeated two additional times, which was sufficient to elute the bulk of HF from the QAE-Sephadex gel. The four filtrates were combined, mixed at 4°C with 390 g/L of solid ammonium sulfate (ie, to 60% saturation), and the precipitate that formed was allowed to settle overnight.

The mixture was centrifuged at 12,500 g for 20 minutes at 4°C and the supernatant fluid decanted. The precipitate was dissolved in as little Tris buffer as possible and dialyzed against running tap water for three hours and against Tris buffer at 4°C overnight. The dialyzed sample, approximately 50 to 100 mL, was centrifuged at 900 g for ten minutes at 2°C to remove undissolved matter. The supernatant fraction was then applied to a 15- to 25-mL packed volume column of popcorn-agarose, equilibrated with barbital-saline-Polybrene buffer, in a 60-mL polypropylene syringe, supported by nylon mesh that had been rinsed in hexadimethrine bromide (10 mg/mL water). The column was washed with 20 mL of the same buffer, and then eluted with ammonium acetate-sodium chloride buffer (pH 6.8), collecting 1.5-mL fractions in polystyrene tubes at 4°C overnight. Tubes containing HF were identified by coagulant assays. The approximately 50 to 80 mL of eluate that had appreciable HF coagulant properties was stored in polystyrene tubes at −70°C until used.

The eluate was dialyzed overnight at 4°C against acetate-Polybrene buffer and applied at the same temperature to a 1.5 x 30-cm column of SP-Sephadex that had been equilibrated in the same buffer. The column was eluted with a linear gradient of 200 mL of acetate buffer in the proximal chamber and 200 mL of the same buffer containing 0.5 mol/L sodium chloride in the distal chamber, collecting 2-mL fractions in polystyrene tubes. Neither of these buffers contained hexadimethrine bromide. Tubes containing HF were identified by coagulant assays. Those fractions with the highest activity, approximately 40 mL, were pooled and stored at −70°C in small aliquots in polypropylene containers.

Coagulant assays of clotting factors were performed in uncoated glass tubes by modifications of the activated partial thromboplastin time and prothrombin time referred to earlier. All samples were diluted in barbital-saline-albumin buffer to provide stability during the assays. Coagulant assays for activated HF were performed by the same technique, using polystyrene tubes (internal diameter 10 mm) coated with Surfasil and omitting kaolin from the kaolin-phospholipid mixture. Total and activated HF were also assayed by an amidolytic method, as previously reported, using a concentration of HF of 0.004 coagulant units per mL in the enzyme-substrate mixture. Plasminogen was assayed by amidolysis of H-D-valyl-L-leucine-p-nitroanilide dihydrochloride (S2251), as described earlier.

The protein concentration at each step was measured by Lowry's method. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of reduced and unreduced samples of purified HF was performed as described earlier and calibrated against a mixture of proteins with defined molecular weights (Electrophoresis Calibration Kit, Pharmacia).

## RESULTS

Batch adsorption with QAE-Sephadex gel and subsequent precipitation with ammonium sulfate reduced the protein content of plasminogen-depleted plasma to about one fifth to one seventh that of the original plasma with about a 40% loss of HF titer (Table 1). Subsequent filtration of the partially purified HF through popcorn-agarose gel resulted in a further loss of about 30% of the HF content of the original plasma, an estimate that may have been exaggerated by the effect of inhibitors in the eluting buffer upon the coagulation assay; there was an increase in the specific activity to about 1,300-fold compared to the starting plasma (Fig 1). This preparation was contaminated with a high molecular weight species of protein that could not be separated readily on Sephadex gels. It was therefore chromatographed upon SP-Sephadex gel (Fig 2) without appreciable loss of HF titer; the eluted HF had been purified about 5,000-fold over the starting plasma and in different preparations had a specific activity of 50.0 to 67.1 U/mg protein with yields of

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**Table 1. Purification of Hageman Factor**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (mL)</th>
<th>Activity (U/mL)</th>
<th>Total Units</th>
<th>Protein (mg/mL)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (U/mg Protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma dil 50%</td>
<td>600</td>
<td>0.42</td>
<td>252</td>
<td>32.0</td>
<td>19,200</td>
<td>0.013</td>
<td>100</td>
</tr>
<tr>
<td>Lys agar ads plasma</td>
<td>660</td>
<td>0.40</td>
<td>264</td>
<td>24.0</td>
<td>15,840</td>
<td>0.017</td>
<td>100</td>
</tr>
<tr>
<td>Dial amm suff ppt</td>
<td>90</td>
<td>1.76</td>
<td>158</td>
<td>39.5</td>
<td>3,555</td>
<td>0.045</td>
<td>63</td>
</tr>
<tr>
<td>PCI eluate</td>
<td>60</td>
<td>1.42</td>
<td>85*</td>
<td>0.109</td>
<td>6.54</td>
<td>13.0</td>
<td>37*</td>
</tr>
<tr>
<td>SP Seph pool</td>
<td>39</td>
<td>2.56</td>
<td>100</td>
<td>0.044</td>
<td>1.72</td>
<td>58.2</td>
<td>40</td>
</tr>
</tbody>
</table>

Abbreviations: Lys agar plasma, lysine-agarose-adsorbed, diluted plasma; Dial amm suff ppt, ammonium sulfate precipitate of lys agar plasma after adsorption to and elution from QAE-Sephadex after dialysis against Tris buffer; PCI eluate, popcorn inhibitor-agarose eluate of ammonium sulfate precipitate after dialysis; SP Seph pool, pooled eluates of PCI eluate adsorbed to SP-Sephadex gel.

*This may have been an underestimate because of the presence of inhibitors in the buffer.
Table 2. Assays of Purified Hageman Factor for Clotting Factors and Plasminogen

<table>
<thead>
<tr>
<th>Factor</th>
<th>Clotting Time (sec)</th>
<th>U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled Plasma</td>
<td>33.4</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Purified HF†</td>
<td>35.0</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

†Clotting time of purified HF (2.6 U/mL), diluted 20-fold, in specific assays for the listed clotting factors.

Table 3. Spontaneous Amidolytic Activity of Purified Hageman Factor

<table>
<thead>
<tr>
<th>Test Mixture</th>
<th>Amidolytic Activity (nmol pNA/mL/60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF + ellagic acid</td>
<td>26.9</td>
</tr>
<tr>
<td>HF + buffer</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Amidolytic activity of a mixture of 0.05 mL HF (0.05 U/mL 0.25% bovine albumin in barbital-saline buffer, 0.1 mL Dulbecco’s phosphate-buffered saline and 0.05 mL 2 × 10⁻⁴ mol/L ellagic acid in barbital-saline buffer, incubated at 37 °C in 10 × 75-mm polystyrene tubes for 60 min, after which 1.0 mL 5 × 10⁻⁴ mol/L S2302 was added and incubation was continued for 60 min. The reaction was stopped and the amount of o-nitroaniline (pNA) released was measured spectrophotometrically at 405 nm.

Fig 1. Filtration of partially purified HF through popcor nagarose gel. 58 mL of ammonium sulfate-precipitated fraction (114 U HF) was applied to a 22-mL column of popcor nagarose equilibrated with barbital-saline-Polybrene buffer. Elution with 0.1 mol/L ammonium acetate buffer (pH 6.8) containing 1.0 mol/L sodium chloride and 100 mg/L hexadimethrine bromide was begun at Tube 1 of the Figure, collecting 1.5-mL fractions. The titer of HF, estimated by specific coagulant assay, is represented in terms of clotting times. The contents of tubes 30 through 66 were pooled (>).

Fig 2. Chromatography of partially purified HF through SP-Sephadex gel. 76 mL of popcorn-agarose eluate (76 U HF) was applied to a 1.5 × 30-cm column of SP Sephadex gel equilibrated with 0.05 mol/L sodium acetate buffer (pH 5.2) in 0.15 mol/L sodium chloride. A linear gradient of this buffer and the same buffer containing 0.50 mol/L sodium chloride was begun at Tube 1 of the Figure, collecting 2.0-mL fractions. The titer of HF, estimated by specific coagulant assay, is represented in terms of clotting times. The contents of tubes 104 through 122 were pooled (>).

Fig 3. SDS-PAGE of purified HF. A volume of 50 μL HF (310 μg/mL, specific activity 67 U/mg protein) was applied to (A) and (B); the HF in (A) was unreduced, and that in (B) was reduced. Protein markers (C), whose mol wts are depicted on the right, were tested after reduction.

33% to 40% of the HF content of the starting plasma. It was devoid of other clotting factors and plasminogen (Table 2). The HF was less than 0.1% in the activated form in coagulant assays, and had no detectable amidolytic properties unless activated by ellagic acid (Table 3). Usually a single band was visible upon SDS-PAGE of reduced and unreduced HF; the molecular weight appeared to be approximately 75,000 kD in comparison to a series of protein standards (Fig 3). In one preparation, the protein band appeared to be a doublet, as was occasionally observed in preparations made by earlier methods. Preparation of HF by the method described required about ten working days. The coagulant...
HF titer was unaltered by storage in polypropylene containers at −70 °C for 30 days.

**DISCUSSION**

Hojima et al. reported that a protein derived from sweet corn, apparently identical to one known to inhibit trypsin, inhibited activated HF. This agent, derived at Hojima’s suggestion from popcorn, appeared to inhibit the activation of HF from its precursor. Kambhu et al. demonstrated that the popcorn inhibitor, rendered insoluble by binding to cyanogen bromide–agarose gels, adsorbed the precursor form of HF from plasma. The HF could be eluted gradually from the gel by washing with barbital-saline buffer, and much more efficiently by the same buffer containing 1.0 mol/L sodium chloride. The preparations obtained, however, had low specific activity and were unsuitable for experimental use.

The technique described in the present report took advantage of this unusual property of the popcorn inhibitor, and made possible a major simplification of the procedure for purification of HF. Speed was also achieved by initial purification of HF by batch elution of plasminogen-depleted plasma on QAE Sephadex. The eluate was further purified by chromatography on popcorn-agarose, but was still contaminated with a high molecular weight protein that was readily removed by SP-Sephadex chromatography. The final product was essentially all in the precursor form, as assessed by coagulant and amidolytic assays.

The procedure described, then, allowed much more rapid purification of HF than did earlier methods, and the product was more reliably obtained in the precursor form.

**REFERENCES**

4. Zimmerman TS, Ratnoff OD, Powell AE: Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand’s disease with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against antihemophilic factor. J Clin Invest 50:244, 1971
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