Sephadex-ellagic acid gels have amidolytic properties.

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PREPARATIONS of purified Hageman factor (HF, factor XII) that have been exposed to Sephadex-ellagic acid gels is a single-chain species (HFm) with amidolytic properties for the synthetic substrate H-D-phenylalanyl-L-pipeocyl-L-arginine p-nitroanilide. Earlier we reported that amidolysis was suppressed by incubation of HFm with specific antisera. The present study provides additional evidence that the amidolytic properties of preparations of HFm are ascribable to this substance through an examination of a number of protease inhibitors. HFm's amidolytic properties were inhibited by α2-plasmin inhibitor, antithrombin III in the presence of heparin, and Cl esterase inhibitor (Cl-INH). Additionally, it was inhibited by popcorn inhibitor, leupeptin, hexadimethrine bromide, proline sul fate, dansyl-arginine N-(3-ethyl-1.5-pentanediyl) amide (DAPA), diisopropylphosphofluoridate (DFP), aprotinin, and at excessively high concentrations, soybean and lima bean trypsin inhibitors. The spectrum of action of agents that did or did not inhibit HFm supports the view that amidolysis by preparations of HFm is attributable to this enzyme. In general, the enzymatically active carboxy-terminal fragment of HF (HFc) was inhibited by the same agents that inhibited HFm, but aprotinin, protamine sulfate and hexadimethrine bromide were more effective against HFtm than HFm, while the reverse was true of lima bean trypsin inhibitor.

On the average, the diluted HF hydrolyzed 1.14 nmole S2238/μg HF/ml/min.

HFm was prepared as described by mixing HF in the presence of bovine albumin with Sephadex-ellagic acid gels. The HFm, in barbital-saline buffer containing 0.05% bovine albumin, was separated by centrifugation. It possessed, on the average, 0.13 ± S.D. 0.04 U/ml (4.6 μg/ml) of total HF, and 0.03 ± 0.01 U/ml (1.1 μg/ml) of coagulant HF, as tested respectively by a modified partial thromboplastin time technique in the presence of kaolin or in the absence of a clot-promoting surface; on the average, HFm hydrolyzed 2.1 nmole S2238/μg HF/ml/min (see below).

HFc was prepared by tryp tic digestion of purified HF, as reported earlier. The preparations used possessed a single species with a MW of about 30,000, as estimated by SDS-PAGE, and contained 17 to 47 μg of protein per ml of barbital-saline buffer. Before use, HFc was further diluted in the same buffer to 2.7 (SD ± 1.3) μg/ml.

Materials and Methods

Purified HF, prepared as previously described, was a single-chain species, as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) after reduction, approximately 3-5 μg protein was tested in this assay. It was depleted of other known factors of the contact-activated clotting system, including prekallikrein, HMW kininogen, PTA, and plasminogen. Two preparations of HF were used, with specific activities of 46 and 113 U/mg protein, as measured in clotting assays; one unit of HF is that amount found in 1 ml of a pool of 24 plasmas derived from normal male subjects.

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Inhibition of enzymatically active fragments of HF (HFm) by the agents tested paralleled inhibition of HFm.

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the gift of Dr. T. Takaya, the Protein Research Foundation, Osaka, Japan; and dansylarginine N-(3-ethyl-1,5-pentanediyl) amide (DAPA), was the gift of M. E. Nesheim, Mayo Clinic, Rochester, Minn. Except for DFP, all inhibitors were dissolved in or diluted as indicated in barbital-saline buffer.

Barbital-saline buffer (pH 7.5) was 0.025 M barbital in 0.125 M sodium chloride. Tris-imidazole-saline buffer (pH 8.2) was 0.025 M tris (hydroxymethyl)aminomethane (Sigma), 0.025 M imidazole (Matheson, Coleman and Bell, Norwood, Ohio) and sufficient sodium chloride to provide an ionic strength of 0.15.

Inhibition of amidolysis by plasma inhibitors of proteolysis was tested by incubating 0.15 ml of HF or HF1 for 30 min at 37°C with 0.1 ml of serial dilutions of the reagents to be tested in barbital-saline buffer, or buffer alone. Thereafter, 1.0 ml of 0.5 mM S2238 and 0.1 ml of serial dilutions of the reagents to be tested in barbital-saline buffer was added. Only this concentration of DFP was tested.

The optical density was compared to that of a standard solution of p-NA.

The effect of other inhibitors (except DFP) was tested in the same manner except that the preliminary incubation period was 10 min. DFP, 0.12 M in absolute ethanol, 2.55 µl, was added to 0.15 ml HF or HF1 to bring the DFP concentrate to 2 × 10⁻³ M, and the mixture was incubated for 10 min. Thereafter 97.45 µl of barbital-saline buffer was added. Only this concentration of DFP was tested.

The concentrations of HF and HF1 were used to select concentrations of 0.3 ml glacial acetic acid. The amount of p-nitroaniline (p-NA) released was read at 405 nm in 10 mm cuvettes in comparison to a blank in which acetic acid was added before addition of substrate. The optical density was compared to that of a standard solution of p-NA.

RESULTS

The amidolytic activity of HF was readily inhibited by α1-antiplasmin inhibitor, CI-INH and a mixture of antithrombin III and heparin (Table 1). Approximately the same concentrations inhibited HF1. In contrast, neither antithrombin III in the absence of heparin, α1-macroglobulin nor α1-antitrypsin inhibited HF or HF1 at the concentrations tested (Table 3).

Amidolysis by both HF and HF1 was inhibited by 2 × 10⁻³ M DFP; lesser concentrations were not tested (Table 2). Both enzymes were comparably inhibited by popcorn inhibitor, leupeptin, and DAPA. In contrast, relatively more hexadimethrine bromide, protamine sulfate, and aprotinin were needed to induce inhibition of HF than HF1. SBTI and LBTI were inhibitory only at very high concentrations.

Several other protease inhibitors did not block amidolysis by HF or HF1 under the conditions examined (Table 3). In an earlier study, we noted that amidolysis by HF and HF1 was enhanced by addition of high molecular weight kininogen, albumin, cytokrome C, and, to a much lesser extent, IgG. The degree of enhancement was much greater for HF1 than for HF, presumably because the latter preparation already contained 0.5 mg albumin/ml (see Materials and Methods). In the present study, α1-antitrypsin, α1-macroglobulin, ovomucoid and SBTI enhanced amidolysis by HF and HF1.

DISCUSSION

Hageman factor (HF, factor XII) that has been exposed to Sephadex-ellagic acid gels (HF) hydrolyses the synthetic amide H-D-phenylalanyl-L-pipercollyl-L-arginine p-nitroanilide (S2238). Revak et al. provided evidence that, in plasma, activation of HF depends upon its scission, first internally within a disulfide loop and then into two portions, an amino-terminal fragment of MW 28,000; the latter bears the enzymatically active group.

Table 2. Nonplasma Inhibitors of Amidolysis by HF and HF1

<table>
<thead>
<tr>
<th>Agent</th>
<th>HF</th>
<th>HF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Popcorn inhibitor</td>
<td>3.2 µg/ml</td>
<td>5.0 µg/ml</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>27 µg/ml</td>
<td>22 µg/ml</td>
</tr>
<tr>
<td>Hexadimethrine bromide</td>
<td>130 µg/ml</td>
<td>12 µg/ml</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>140 µg/ml</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>DAPA</td>
<td>5 x 10⁻⁴ M</td>
<td>4 x 10⁻⁴ M</td>
</tr>
<tr>
<td>DFP</td>
<td>&lt;2 x 10⁻³ M</td>
<td>&lt;2 x 10⁻³ M</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>4000 U/ml</td>
<td>1000 U/ml</td>
</tr>
<tr>
<td>SBTI</td>
<td>&gt;8 mg/ml</td>
<td>&gt;8 mg/ml</td>
</tr>
<tr>
<td>LBTI</td>
<td>2 mg/ml</td>
<td>8 mg/ml</td>
</tr>
</tbody>
</table>

*Concentration in enzyme-inhibitor mixture before addition of substrate.
†Lesser amounts augmented amidolysis.
‡Only concentration tested.

Table 3. Some Agents Not Inhibiting Amidolysis by HF and HF1

<table>
<thead>
<tr>
<th>Agent</th>
<th>HF</th>
<th>HF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin III</td>
<td>0.1 mg/ml</td>
<td>0.1 mg/ml</td>
</tr>
<tr>
<td>α1-Macroglobulin</td>
<td>0.1 mg/ml</td>
<td>0.1 mg/ml</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>0.04 mg/ml</td>
<td>0.16 mg/ml</td>
</tr>
<tr>
<td>Plasminostreptin</td>
<td>0.1 mg/ml</td>
<td>0.08 mg/ml</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>1.6 mg/ml</td>
<td>0.4 mg/ml</td>
</tr>
<tr>
<td>Hirudin</td>
<td>1000 U/ml</td>
<td>1000 U/ml</td>
</tr>
<tr>
<td>Tranexamic acid</td>
<td>0.4 mg/ml</td>
<td>0.4 mg/ml</td>
</tr>
</tbody>
</table>

*Concentration in enzyme-inhibitor mixture before addition of substrate.
†Enhancement of amidolysis.
‡Higher concentrations enhanced amidolysis.

NOTE

The optical density was compared to that of a standard solution of p-NA.
HF<sub>a</sub>, in contrast, is a single-chain species with a MW of about 80,000. The possibility exists that its amidolytic properties reflect the unsuspected presence of other plasma proteases that are either themselves amidolytic or might be responsible for scission of HF<sub>a</sub> subsequent to its incubation with its substrates. Earlier we reported that amidolysis was suppressed by incubation of HF<sub>a</sub> with specific antiserum, supporting the specificity of the observed enzymatic activity. The present study provides additional evidence that the amidolytic properties of preparations of HF<sub>a</sub> are ascribable to this substance through an examination of the inhibitory properties of a number of agents chosen for their possibly differentiating properties.

The clearest separation of HF<sub>a</sub> from plasma proteases other than HF was seen in its inhibition by popcorn inhibitor. Hojima and his associates recently reported that one or more proteins derived from sweet corn or popcorn inhibited amidolysis by HF<sub>a</sub>. These agents were without effect upon α-thrombin, activated Stuart factor (factor Xa), or plasma kallikrein. Additionally, popcorn inhibitor does not block the actions of activated PTA (factor Xla), activated Christmas factor (factor IXa) or factor VII, as tested in clotting assays, nor of the activated form of the first component of complement (C<sub>1</sub>), as determined by esterolysis of N-acetyl-L-tyrosine ethyl ester.

Although these studies tell us that preparations of HF<sub>a</sub> possessed unique amidolytic properties, they do not rule out the presence of contaminating enzymes. Studies of other inhibitors, however, demonstrated that the amidolytic properties of HF<sub>a</sub> were not blocked by a variety of substances that inhibit other known plasma proteases. Thus plasminostreptin, an inhibitor of plasmin found in cultures of Streptomyces antifibrinolyticus, and hirudin, which specifically blocks the action of thrombin and probably activated Christmas factor, were without effect upon amidolysis by HF<sub>a</sub>. Similarly, α<sub>_1</sub>-antitrypsin, which inhibits activated PTA, α<sub>_2</sub>-macroglobulin, which inhibits plasma kallikrein and plasmin, and tranexamic acid, which inhibits plasmin were without effect upon HF<sub>a</sub>. Soybean trypsin inhibitor (SBTI), which in small concentrations blocks the actions of plasmin, plasma kallikrein and activated Stuart factor had only minimal activity against HF<sub>a</sub> at excessively high concentrations.

These studies do not rule out contamination of HF<sub>a</sub> with C<sub>1</sub>, but preparations of HF<sub>a</sub> do not hydrolyze N-acetyl-L-tyrosine ethyl ester, a specific substrate of C<sub>1</sub> (unpublished observations). Nor do they rule out contamination with factor VII, whose amidolytic properties were not tested.

A number of other agents that inhibited the action of HF<sub>a</sub> also inhibit other proteases, and thus were not helpful in the present context. For example, although hexadimethrine bromide and protamine sulfate inhibited HF<sub>a</sub>, these agents also block the clot-promoting properties of activated Stuart factor.

In general, HF<sub>a</sub> was inhibited only by those agents that inhibited HF<sub>a</sub>. Notably, however, aprotinin, protamine sulfate and hexadimethrine bromide were more effective against HF<sub>a</sub> than HF<sub>a</sub>, while the reverse was true for LBTI.

In sum, the inhibitory spectrum of HF<sub>a</sub> appeared to differentiate this enzyme from other plasma proteases, in agreement with the view that the amidolytic properties of this preparation are attributable to a single-chain, activated species of Hageman factor.

ACKNOWLEDGMENT

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Studies on the inhibition of ellagic acid-activated Hageman factor (factor XII) and Hageman factor fragments

OD Ratnoff