Inactivation of Various Proteinase Inhibitors and the Complement System in Human Plasma by the 56-Kilodalton Proteinase from Serratia marcescens

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Received 11 November 1988/Accepted 20 February 1989

The interaction of the 56-kilodalton (kDa) proteinase from Serratia marcescens with human plasma activated C1 (C1) inhibitor, α2-antiplasmin, and antithrombin III was investigated. The 56-kDa proteinase was not affected by these inhibitors; on the contrary, all the inhibitors were inactivated by the 56-kDa proteinase within 2 to 6 h. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that all three inhibitors showed decreases in molecular weight of approximately 8,000 to 10,000 as a result of proteolytic cleavage by the 56-kDa proteinase. The 56-kDa proteinase also inactivated serum complement within 2 to 6 h.

Plasma proteinase inhibitors play important roles in modulating the activity of the proteinase cascade system involved in different defense and clotting mechanisms. Activated C1 (C1) inhibitor, α2-proteinase inhibitor (α1-PI), α2-macroglobulin (α2-M), antithrombin III (AT-III), and α2-antiplasmin (α2-AP) are involved in several of these systems. C1 inhibitor is the main inhibitor of C1s, C1r, kalikrein, and factor XIIa (32). Kalikrein is involved in the inflammatory response during bacterial infection, releases kinin from kinogen, and acts on plasmingen to release plasmin (2, 13, 14, 27). Plasmin also activates the Hageman factor and prekalikrein (16). α2-AP binds with and inactivates plasmin (3) and slowly inactivates kalikrein, factor Xa, and thrombin in a purified system (26). AT-III inhibits thrombin, factor IXa, and factor Xa (29).

The opportunistic pathogen Serratia marcescens causes destructive corneal infections, pneumonia, and urinary tract infections (5, 8, 11, 19). The pathogen secretes three different proteinases, of which the 56-kilodalton (kDa) proteinase is the major one, as described previously (5, 12, 17). The enzymes cause (i) liquefactive necrosis of the cornea (5, 19); (ii) inactivation of α1-PI (18, 22, 28); (iii) degradation of immunoglobulins (immunoglobulin G and immunoglobulin A), fibronectin, and other serum proteins (17, 18, 21); (iv) a potent toxic effect on fibroblasts which was mediated by internalization of a transitory stable complex of proteinase-α2-M via the α2-M receptor (10); and (v) activation of the Hageman factor-kallikrein system generating kinin (6, 13, 14).

We report here an investigation of the interaction of the 56-kDa proteinase with the plasma proteinase inhibitors that are known to be pathophysiologically important as described above. In addition, inactivation of serum complement by this proteinase, which is known to be involved in host defense against infection, is also described.

S. marcescens 56-kDa proteinase was purified from the culture supernatant by ammonium sulfate precipitation, DEAE-cellulose ion-exchange chromatography, and gel filtration (Sephadex G-100) chromatography to homogeneity as described previously (12). The proteinase was homogenerous after sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Inhibition of the 56-kDa proteinase by human plasma C1 inhibitor, α2-AP (Protogen AG, Weidenmattweg, Switzerland), or AT-III (Boehringer Biochemia GmbH, Mannheim, Federal Republic of Germany) was examined at an enzyme/inhibitor (E/I) molar ratio of 1:1 to 1:4 in 0.05 M Tris hydrochloride buffer (pH 7.5) at 37°C for 30 min to 20 h by the fluorescence polarization method using fluorescein isothiocyanate-gelatin as the substrate as described previously (9). The proteolytic activity of the 56-kDa proteinase was unaffected by any of these inhibitors even though the incubation was continued for 20 h (data not shown), which indicated that these inhibitors did not affect the 56-kDa proteinase even at an excess concentration of inhibitors.

Inactivation of purified C1 inhibitor (M, 105,000) and α2-AP (M, 70,000) by the 56-kDa proteinase was tested by their plasmin inhibition activity. Similarly, inactivation of purified AT-III (M, 60,000) by the 56-kDa proteinase was assayed by inhibition against thrombin. To 180 μg of each inhibitor, different amounts of the 56-kDa proteinase ranging from 0.96, 1.92, and 3.84 μg for C1 inhibitor, 5.76 and 14.4 μg for α2-AP, and 3.36 and 6.72 μg for AT-III, were mixed in a total volume of 450 μl of either 0.1 M phosphate buffer (pH 7.3) (for C1 inhibitor and α2-AP) or 0.05 M Tris hydrochloride buffer in 0.1 M NaCl and 0.1% polyethylene glycol (pH 7.4) (for AT-III). Heparin enhances the rate of reaction of AT-III and thrombin severalfold (4); thus 0.5 μg of heparin per ml was added before adding the 56-kDa proteinase to AT-III. The mixtures were incubated at 37°C for 0 to 6 h. After different time intervals, a 50-μl aliquot was removed to which was added a known concentration of human plasmin (for C1 inhibitor and α2-AP) and a known concentration of bovine thrombin (for AT-III), followed by an incubation period of another 5 min. The residual plasmin or thrombin activity was then measured by using the synthetic substrate t-butyloxycarbonyl (BOC)-Glu-Lys-Lys-4-methyl-coumaryl-7-amide (MCA) or BOC-Val-Pro-Arg-MCA (Peptide Institute, Inc., Minoh, Osaka, Japan), respectively, by using a fluorescence spectrophotometer with excitation at 380 nm and emission at 441 nm. With this concentration and incubation time, the 56-kDa proteinase had no action on plasmin.

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TABLE 1. Inactivation of human plasma proteinase inhibitors by S. marcescens 56-kDa proteinase

<table>
<thead>
<tr>
<th>Incubation time (h) with 56-kDa proteinase</th>
<th>% Inactivation of the following inhibitor at the indicated E/I ratio</th>
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<tbody>
<tr>
<td></td>
<td>C1 inhibitor&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1&lt;sup&gt;i&lt;/sup&gt;</td>
<td>1:25</td>
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<tr>
<td>0.5</td>
<td>0</td>
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<tr>
<td>1</td>
<td>0</td>
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<tr>
<td>2</td>
<td>0</td>
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<td>4</td>
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<sup>a</sup> Determined by human plasmin inhibitory activity of C1 inhibitor and α1-AP, using the synthetic substrate for plasmin BOC-Glu-Lys-Lys-MCA. See text for details.

<sup>b</sup> Determined by bovine thrombin inhibitory activity of AT-III, using the synthetic substrate for thrombin BOC-Val-Pro-Arg-MCA. See text for details. Heparin (0.5 μg/ml) was added in the assay mixture.

<sup>i</sup> Inhibitor alone without the 56-kDa proteinase was incubated and plasmin or thrombin inhibition assay was used as a control experiment. Virtually no inhibitory activity was lost.

or thrombin. The 56-kDa proteinase did not hydrolyze thrombin substrate but did very slowly hydrolyze plasmin substrate (BOC-Glu-Lys-Lys-MCA). Thus, the background value of BOC-Glu-Lys-Lys-MCA was subtracted from a total value to give a net value. Inhibitors alone (without the 56-kDa proteinase) were incubated at the same incubation period as a control experiment. Inhibitory activity of C1 inhibitor, α1-AP, or AT-III against plasmin or thrombin did not change even after 6 h of incubation in the absence of the 56-kDa proteinase (Table 1). The incubation of C1 inhibitor, α1-AP, or AT-III with the 56-kDa proteinase caused a rapid decrease of inhibitory activity against plasmin or thrombin (Table 1). Within 4 h at 37°C, the 56-kDa proteinase inactivated the C1 inhibitor about 85 and 65% when E/I molar ratios of 1:25 and 1:50 were used, respectively. About 90 and 50% of inhibitory activity of α1-AP was destroyed within 6 h by E/I molar ratios of 1:10 and 1:25, respectively. About 90 and 50% of inhibitory activity of AT-III were also lost within 2 h at E/I molar ratios of 1:25 and 1:50, respectively, in the presence of heparin (Table 1).

To determine whether the inactivation had occurred by limited proteolysis, enzyme and inhibitors E/I at molar ratios of 1:10 to 1:50 were incubated at 37°C for 3 to 7 h and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Weber and Osborn (31). The results shown in Fig. 1 indicated that the proteinase converted native C1 inhibitor, α1-AP, or AT-III into an inactive smaller molecular mass (105,000 to 95,000, 70,000 to 62,000, or 60,000 to 52,000 Da), respectively.

We also tested the effect of the 56-kDa proteinase on complement by using normal human serum as the source of complement. To 1 ml of normal human serum was added 60 to 80 μg of proteinase; the final volume of 2 ml was made by using glucose-gelatin-0.075 M Veronal buffer (pH 7.5) containing 0.15 mM Ca<sup>2+</sup> and 0.5 mM Mg<sup>2+</sup>. The mixtures were incubated at 37°C, and aliquots were removed at 15-min intervals and diluted in tubes containing 2 ml of the above buffer. The concentration giving 50% hemolysis of sera was determined without enzyme as described by Mayer (15). Briefly, 10<sup>9</sup> sheep erythrocytes (SRBCs) were sensitized with a 200-fold-diluted rabbit anti-SRBC immunoglobulin G (Inter-Cell-Technologies, Inc., Somerville, N.J.). Diluted test sera and control sera were incubated with 10<sup>6</sup> sensitized SRBCs for 60 min at 37°C. Ice-cold saline (5 ml) was then added to all tubes to stop the hemolysis. Unlysed SRBCs were removed by centrifugation (850 × g, 5 min), and the release of hemoglobin into the supernatant fluid was measured spectrophotometrically at 541 nm.

Figure 2 demonstrates that hemolytic activity of the serum alone (control, no proteinase) remained almost the same after 6 h of incubation, whereas the activity decreased progressively with time when the serum was treated with the 56-kDa proteinase. At about 1.7 and 4.2 h, 50% of the hemolytic activity was lost with 80 and 60 μg of proteinase per ml of serum, respectively, and after 3 and 6 h, the hemolytic activity of serum was completely lost (Fig. 2). The amount of α1-M in serum was almost in the stoichiometric amount needed to form the complex with 80 μg of the proteinase, whereas it may be slightly in excess for 60 μg of
proteinase per ml. Thus, the difference of inactivation velocity of complement might have caused the lag period needed to destroy α2-M that was excess over the smaller amount of the proteinase (20). The proteinase-α2-M complex is known to liberate gradually free active proteinase (10, 20).

We then determined the effect of Kunitz-type soybean trypsin inhibitor (SBTI) (Fuji Seiyu Co., Osaka, Japan) on complement inactivation by the 56-kDa proteinase. Mixtures of complement, 56-kDa proteinase, and SBTI (final concentration, 10 μM) were incubated at 37°C for 0 to 6 h, and hemolytic activity was determined. The results showed that the 56-kDa proteinase inactivated complement at almost the same rate as without SBTI (data not shown), which indicated no involvement of plasmin, kallikrein, or other serine proteinases.

We (18, 22) and Virca et al. (28) previously demonstrated that serrial proteinases inactivated α1-PI rapidly at a very low enzyme concentration. We also reported that α2-M was degraded by the 56-kDa proteinase and that the proteinase was internalized via the α2-M receptor after forming an enzyme-inhibitor complex (10, 20). The α2-M in the complex was degraded and the proteinase activity was regenerated in the cell, which resulted in cell killing (10, 20). This indicates an unique toxic mechanism of the proteinase in cells. We showed here that the serrial 56-kDa proteinase is a potent inactivator of C1 inhibitor, α2-AP, and AT-III. This inactivation and degradation of various proteinase inhibitors would affect the activity of endogenous serine proteinases, such as plasmin, and result in severe pathological consequences, such as blood coagulation, tissue destruction, edema formation, and pain. When the 56-kDa proteinase was injected intratracheally to the lung, pulmonary hemorrhage, edema formation, and tissue damage were observed in guinea pigs, which leads some support to this hypothesis (data not shown).

Five major proteinase inhibitors, such as α2-M, α1-PI, C1 inhibitor, α2-AP, and AT-III, participate in regulating various cascade mechanisms: Hageman factor-kallikrein-kinin cascade, fibrinolysis and clotting cascade, complement system, and inflammatory response. These cascades may be highly enhanced when the inhibitors are inactivated. For instance, in disseminated intravascular coagulation, the regulation of clotting and fibrinolysis by these inhibitors is out of control in vivo.

In all these serine proteinase inhibitors (serpins), α1-PI, C1 inhibitor, α2-AP, and AT-III, binding sites, having molecular sizes of 5 to 10 kDa, to the proteinases are located in the COOH-terminal end, and the amino acid sequences of the binding sites are known to be -Met-Ser-, -Arg-Thr-, -Arg-Met-, and -Arg-Ser-, respectively (1, 7, 23–25, 27). The 56-kDa proteinase cleaves between Met-358 and Ser-359 in α1-PI, releasing a 5-kDa fragment, and causes a critical conformation change of the inhibitor (28). In this communication, we showed that the molecular size decreased about 8 to 10 kDa from the intact C1 inhibitor, α2-AP, and AT-III by treatment with the 56-kDa proteinase. Thus, the intact inhibitor molecules seem to be degraded into smaller modified inhibitors that were completely inactive. Because of the sequence homology of the serpins, particularly around the reactive site, we speculate a similar cleavage could have occurred as observed in α1-PI. As to the substrate specificity of the 56-kDa proteinase, we previously showed that the 56-kDa proteinase, like many of the serine proteinases, preferentially cleaved the peptide bonds at the COOH-terminal side of the Arg residue in fibronectin and many synthetic peptides (13, 21).

Ward et al. (30) demonstrated that proteinase from S. marcescens cleaved purified human C3 and C5 to yield leukotactic fragments, but they did not determine the functional activity of complement in serum after treatment with serrial proteinase. Here we demonstrated that the 56-kDa proteinase inactivated the complement system. SBTI did not inhibit the inactivation of complement by the 56-kDa proteinase, whereas SBTI could inhibit kallikrein, which is released after activation of the Hageman factor. Kallikrein also releases plasmin from plasminogen. This experiment thus showed that the 56-kDa proteinase directly affected complement but that this complement inactivation was not mediated through the activation of Hageman factor or plasminogen. The inactivation of serum complement system by this proteinase may further aggravate the defense functions because complement is involved in the phagocytosis of foreign particles and killing of bacteria.

This work was supported in part by a grant-in-aid for scientific research from Monbusho (the Ministry of Education, Science, and Culture) of Japan and by Yakult Honsha Co., Tokyo.

We thank Judith B. Gandy for editing the manuscript and M. Fuji for typing the manuscript.

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