Activation of Hageman Factor and Prekallikrein and Generation of Kinin by Various Microbial Proteinases*

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Activation of the Hageman factor-kallikrein-kinin system by serratial 56-kDa proteinase was previously demonstrated (Matsumoto, K., Yamamoto, T., Kamata, T., and Maeda, H. (1984) J. Biochem. (Tokyo) 96, 739–749; Kamata, R., Yamamoto, T., Matsumoto, K., and Maeda, H. (1985) Infect. Immun. 48, 747–753). To investigate whether the activation of the system is specific for 56-kDa proteinase or is found similarly with other microbial proteinases, 11 proteinases of microbial origins were studied; the 56-kDa proteinase was the control. For in vitro studies, activation of guinea pig Hageman factor and prekallikrein was examined in purified systems as well as in plasma as a yzomen source. Specific antibodies and inhibitors confirmed the activation steps of the cascade. In the in vivo study the enhancement of vascular permeability in guinea pig skin and its sensitivity to inhibitors of activated Hageman factor, plasma kallikrein, or a kininase were examined. The results from the in vivo experiments were consistent with those in vitro. Taking all the data together, we classified the 11 microbial proteinases into three groups as follows: 1) Serratia marcescens 56-, 60-, and 73-kDa proteinases, Pseudomonas aeruginosa alkaline proteinase and elastase, and Aspergillus melleus proteinase (this group activated Hageman factor but not prekallikrein); 2) Vibrio vulnificus proteinase, subtilisin from Bacillus subtilis, and thermolysin from Bacillus steatorrhophilus (this group activated both Hageman factor and prekallikrein); 3) Streptomyces caesipotosus proteinase and V8 proteinase from Staphylococcus aureus (this group activated neither Hageman factor nor prekallikrein, but generated kinin from high molecular weight kininogen directly).

Evidence for an indispensable role for microbial proteinases in the initiation and development of various infectious diseases has been accumulating (1–5). We have shown that the clinical severity of keratitis caused by Serratia marcescens depends on the production of tissue-destructive proteinases (2, 4). The proteinases produced by this organism cause (i) liquefactive necrosis when injected into the cornea (2, 4); (ii) degradation of immunoglobulins (IgG, IgA), fibronectin, α2-macroglobulin (α2M), and α-l-proteinase inhibitor (6–10); (iii) severe cytotoxicity after internalization of the proteinase-α2M complex through α2M receptors on the cell surface (11); and (iv) a vascular permeability enhancement reaction by activation of the Hageman factor-kallikrein-kinin system (12–14), and simultaneous production of pain at the site of infection.

We have also demonstrated that some of the microbial proteinases (e.g. Pseudomonas proteinases) cause an acute liquefactive necrosis in the guinea pig cornea and a vascular permeability enhancement reaction in guinea pig skin similar to serratal 56-kDa proteinase (4). However, details of the mechanism of the permeability enhancement by these microbial proteinases other than 56-kDa proteinase remain to be clarified. A proteinase from Vibrio vulnificus, which causes severe systemic hemorrhage, necrosis, and edema, was found to activate the kallikrein-kinin system, resulting in vascular permeability enhancement (15). However, the direct target yzomen of this proteinase in plasma remains to be clarified. In the present study we examined whether the Hageman factor activation described above is a specific property of 56-kDa proteinase or common among many microbial proteinases. For this purpose we chose several microbial proteinases including those from pathogenic and non-pathogenic microbes and examined their ability to activate the Hageman factor-dependent pathway in vitro and in vivo.

MATERIALS AND METHODS

Substances—Hageman factor was purified from guinea pig plasma according to our previous publication (16). Prekallikrein was prepared from guinea pig plasma, and kallikrein was purified after activation of prekallikrein with activated guinea pig Hageman factor as described previously (17, 18). High molecular weight kininogen was purified from guinea pig plasma according to our previous publication (19). Antibodies against guinea pig Hageman factor and prekallikrein were raised in rabbits. The anti-Hageman factor F(ab')₂ antibody was immunopurified with the guinea pig Hageman factor immobilized on Sepharose 4B beads after purifying IgG and treating with pepsin as described previously (20). The anti-prekallikrein F(ab')₂ antibody was purified by gel filtration after pepsin treatment of the IgG fraction of the antiserum as described (18). These antibodies specifically inhibited guinea pig activated Hageman factor or kallikrein, respectively (18, 20). Corn trypsin inhibitor (CTI) was prepared as described by Hofjima et al. (21). Serratia 56-, 60-, and 73-kDa proteinases were purified from a clinical isolate of S. marcescens as described previously (22). V. vulnificus proteinase (50 kDa) was purified from the culture medium of V. vulnificus as described previously (23). Crystallized

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1 The abbreviations used were: α2M, α2-macroglobulin; CTI, corn trypsin inhibitor; STI, soybean trypsin inhibitor (Kunitz type); SQ 28881, Ghr-Tsp-Pro-Arg-Pro-Glu-Ile-Pro-OH (kininase inhibitor); MCA, 4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; Z, carboxbenzoxyl.
**Microbial Proteinases and Kinin Generating System**

*Pseudomonas aeruginosa* alkaline proteinase (48 kDa) and *Pseudomonas elastase* (58 kDa) were purchased from Nagase Biochemicals, Osaka, Japan. Thermolysin (37 kDa) produced by *Bacillus stearothermophilus* and *Aspergillus* proteinase produced by *Aspergillus mellus* were obtained from Amano Pharmaceuticals, Nagoya, Japan. Subtilisin produced by *Bacillus subtilis* (27 kDa), V8 proteinase (12 kDa) produced by *Staphylococcus aureus*, and *Streptomyces* caspotoxin proteinases were purchased from Sigma. Soybean trypsin inhibitor (STI) (Kunitz type) was a gift from Fuji Seiyu Co., Osaka, Japan. All proteins and proteinases were homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All peptidyl-4-methylcoumaryl-7-amide (peptidyl-MCA) substrates were purchased from the Peptide Institute, Minoh, Osaka, Japan. Glu-Trp-Pro-Arg-Pro-Glu-Le-Pro-OH (SQ 20881) was a gift from Squibb Institute, Princeton, NJ. An enzyme immunoassay kit for kinin (MARKIT \( \text{Bradykinin} \)) was obtained from Dainippon Pharmaceutical, Osaka, Japan. All other chemicals were purchased from Wako Pure Chemicals Industry, Osaka, Japan.

**Animals**—Albino Hartley guinea pigs of both sexes (400-600 g) were used, and treatments were done under anesthesia with sodium pentobarbital.

**Assay for Proteolytic Activity of Microbial Proteinases**—Fluorescence polarization was employed using fluorescein isothiocyanate-labeled gelatin as the substrate (24).

**Assay for Hageman Factor and Prekallikrein Activation in Plasma**—Guinea pig plasma (100 \( \mu l \)) was mixed with 850-870 \( \mu l \) of 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl and 0.05% polybrene and with 20 \( \mu l \) of the MCA substrate (final concentration 0.1 mM) at 37°C. After addition of 10 \( \mu g \) of a microbial proteinase (in 10-20 \( \mu l \)), liberation of 7-amino-4-methyl-coumarin (AMC) from the fluorogenic substrate (MCA peptides) was continuously recorded with excitation at 380 nm and emission at 441 nm by using a fluorescence spectrophotometer (Hitachi Model 850-40). The fluorogenic substrates Pro-Phe-Arg-MCA and Z-Phe-Arg-MCA were used to assay the release of activated Hageman factor or kallikrein, respectively. The amount of AMC increased in an exponential or hyperbolic fashion during the assay period when the corresponding plasma proteinase zymogens were activated. The rate of hydrolysis of MCA peptides at each time point was calculated from the recorder chart within 5 min.

**Inhibition Studies Using Specific Antibodies and Inhibitors**—To identify which plasma proteinases were generated, the anti-Hageman factor F(ab')\(_2\) or anti-prekallikrein F(ab')\(_2\) antibody (final concentration 0.1 mM each) was added into the plasma which had been pretreated with the respective microbial proteinases for 3-5 min in the same way as described above. After further incubation for 2 min, remaining amidolytic activity of the plasma proteinase(s) was measured by adding the substrate, Pro-Phe-Arg-MCA or Z-Phe-Arg-MCA as described above. A similar experiment was carried out in the presence of CTI or STI (final concentration 0.01 mM) plus microbial proteinase.

**Activation of Hageman Factor by Microbial Proteinases in Vitro**—In this system, the purified Hageman factor (15 \( \mu g/ml \)) in 1 ml of 0.02 M Tris-HCl buffer containing 0.15 M NaCl and 0.05% polybrene, with 20 \( \mu l \) of the MCA substrate (final concentration 0.1 mM) was added into the plasma which had been pretreated with the respective microbial proteinases for 3-5 min in the same way as described above. After further incubation for 2 min, remaining amidolytic activity of the plasma proteinase(s) was measured by adding the substrate, Pro-Phe-Arg-MCA or Z-Phe-Arg-MCA as described above. A similar experiment was carried out in the presence of CTI or STI (final concentration 0.01 mM) plus microbial proteinases in plasma.

**Activation of Prekallikrein by Microbial Proteinases in Vitro**—In this system, the purified prekallikrein (15 \( \mu g/ml \)) was used. The buffer and other procedures were essentially the same as those described in the preceding paragraphs.

**Coactivation of Hageman Factor and Prekallikrein by Microbial Proteinases in Vitro**—In this case, the purified Hageman factor (15 \( \mu g/ml \)), purified prekallikrein (15 \( \mu g/ml \)), and Z-Phe-Arg-MCA were mixed with each microbial proteinase as described in the preceding paragraphs. The release of kallikrein amidolytic activity was then measured as described above.

**Assay of Kinin Generation and Degradation**—A commercial assay kit for bradykinin was used. High molecular weight kininogen (180 \( \mu g/ml \)) in 0.02 M Tris-HCl buffer containing 0.1 M NaCl and 0.1 mg/ml bovine serum albumin (pH 7.4) was incubated with 0.5-10.0 \( \mu l \) of *Staphylococcus* V8 or *Streptomyces* proteinase at 37°C for 10 min. After stopping the reaction with trichloroacetic acid solution (10% final concentration 6%), the kinin content in the supernatant was measured by an enzyme immunoassay according to the instructions of the manufacturer (13). Synthetic bradykinin was used as standard. Synthetic bradykinin was also incubated with these proteinases at 37°C for 10 min, and the remaining bradykinin in the reaction mixture was measured.

**Measurement of Vascular Permeability Enhancement in Guinea Pig Skin**—One hundred microliters of saline containing 1-3 \( \mu g \) proteinase was injected intradermally into the clipped flank of guinea pigs immediately after intravenous injection of 15 mg of Evans Blue/kg body weight (1.5% Evans Blue solution in saline). Permeability was quantitated by the dye extraction method (25). The effects of CTI, STI, SQ 20881, carboxypeptidase B, and antihistamine on the permeability enhancement were determined by *in situ* injection of proteinases that had previously been mixed with these reagents, as described earlier (12).

**Fig. 1**. Generation of kallikrein activity in guinea pig plasma by microbial proteinases and their inhibition by CTI or STI. Plasma (100 \( \mu l \)) in a total volume of 1 ml in 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl, 0.05% Polybrene, with 10 \( \mu g \) of microbial proteinases in the presence or absence of CTI or STI, was incubated with the synthetic substrate for kallikrein Z-Phe-Arg-MCA (Z-FR-MCA). The activity of kallikrein was then continuously measured at 37°C as described under "Experimental Procedures." A, alone; or B or C, *Serratia* 56- to 60-kDa proteinases, respectively; O or D, *Pseudomonas* alkaline proteinase or elastase, respectively; O or E, *Aspergillus* proteinase; O, all above proteinases with CTI or STI (Kunitz type). In B, or O or C, *Vibrio* proteinase alone or with CTI, respectively; or O or D, subtilisin alone or with CTI, respectively; or E, thermolysin or CTI, respectively; O, plasma alone or with V8, or *Streptomyces* proteinase, or all proteinases with STI. The results with *Serratia* 73-kDa proteinase were similar to those with *Serratia* 56-kDa proteinase (see text for details).
Microbial Proteinases and Kinin Generating System

TABLE I

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Pro-Phe-Arg-MCA</th>
<th>Z-Phe-Arg-MCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Anti-HF-Ab¹</td>
<td>Anti-HF-Ab²</td>
</tr>
<tr>
<td>Plasma alone</td>
<td>[0.1]¹</td>
<td>[0.1]¹</td>
</tr>
<tr>
<td>Pseudomonas alkaline</td>
<td>2.4 (85)</td>
<td>2.6 (85)</td>
</tr>
<tr>
<td>Proteinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteasomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alkaline proteinase</td>
<td>2.4 (85)</td>
<td>2.6 (85)</td>
</tr>
<tr>
<td>elastase</td>
<td>1.3 (85)</td>
<td>2.0 (85)</td>
</tr>
<tr>
<td>56-kDa proteinase</td>
<td>5.4 (93)</td>
<td>6.7 (93)</td>
</tr>
<tr>
<td>Aspergillus proteinase</td>
<td>3.8 (89)</td>
<td>4.0 (87)</td>
</tr>
<tr>
<td>Vibrio proteinase</td>
<td>2.8 (75)</td>
<td>3.5 (66)</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>3.8 (76)</td>
<td>4.7 (68)</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>2.4 (77)</td>
<td>2.8 (68)</td>
</tr>
</tbody>
</table>

¹ Plasma incubated with proteinases for 3-5 min, and then anti-Hageman factor or anti-prekallikrein antibody (100 µM, final concentration) was added and allowed to incubate for 2 min and AMC release was measured for another 2 min.
² Plasma was incubated with anti-Hageman factor antibody for 10 min and then proteinases added, and immediately after addition of MCA substrate AMC release was measured.
³ Values in brackets were subtracted as a background.

RESULTS AND DISCUSSION

Hageman Factor Activation and Kallikrein Generation in Plasma—When guinea pig plasma was incubated with the microbial proteinases to examine the generation of kallikrein, hydrolytic activity toward Z-Phe-Arg-MCA (for kallikrein) appeared within 1 min except in the case of V8 and Streptomyces proteinases (Fig. 1, A and B). The Z-Phe-Arg-MCA was hydrolyzed slowly by the 56-kDa proteinase itself and very slowly by other proteinases. Thermolysin, Pseudomonas elastase, V. vulnificus proteinase, and V6 proteinase did not do so. The background rates of hydrolysis of Z-Phe-Arg-MCA by the microbial proteinases were subtracted from the apparent velocity to determine the net generated kallikrein-like activity. The amidolytic velocity increased in a time-dependent manner, thus indicating progressive release of kallikrein-like activity in plasma by these proteinases. The kallikrein-like activity once generated in plasma was inhibited more than 85% by treatment with anti-kallikrein F(ab')₂ antibody (Table I) but not with F(ab')₂ fragment of nonimmunized rabbit IgG (not shown). Furthermore, in the presence of STI a potent inhibitor of kallikrein, the kallikrein-like activity was abolished (Fig. 1, A and B). STI did not inhibit the activity of all microbial proteinases themselves except for subtilisin, V8, or Streptomyces proteinases (not shown). From these facts we concluded that the majority of the Z-Phe-Arg-MCA hydrolytic activity generated in plasma by the microbial proteinases was attributable to plasma kallikrein.

To examine whether kallikrein generation in plasma was caused by activation of Hageman factor, the same experiments as described above were performed but in the presence of anti-Hageman factor F(ab')₂ antibody or an inhibitor against activated Hageman factor, CTI. CTI did not block proteolytic activity of the microbial proteinases except for V8 and Strept-
microbial proteinases, respectively; \( \text{O} \) or \( \Delta \), \text{Pseudomonas} alkaline proteinase or elastase, respectively; \( \Box \), \text{Aspergillus} proteinase; \( \text{O} \), Hageman factor and prekallikrein alone. \( \bullet \), \text{Vibrio} proteinase; \( \Delta \), subtilisin; \( \text{I} \), thermolysin; \( \text{O} \), V8 or \text{Streptomyces} proteinases. Results with \text{Serratia} 73-kDa proteinase were similar to those with \text{Serratia} 56-kDa proteinase. Note that V8 and \text{Streptomyces} proteinases did not release kallikrein, which indicates that neither Hageman factor nor prekallikrein was activated.

Fig. 4. Generation of kallikrein activity in a mixture of purified Hageman factor and prekallikrein during a 3-min incubation at 37°C. The concentrations of both Hageman factor and prekallikrein were 15 μg/ml and that of the proteinases 10 μg/ml. Other conditions were similar to those of Fig. 2. Kallikrein generation was measured immediately after adding Z-Phe-Arg-MCA (Z-FR-MCA) as described in Fig. 1. \( \bullet \) or \( \text{I} \), \text{Serratia} 56- or 60-kDa proteinases, respectively; \( \text{O} \) or \( \Delta \), \text{Pseudomonas} alkaline proteinase or elastase, respectively; \( \Box \), \text{Aspergillus} proteinase; \( \text{O} \), Hageman factor and prekallikrein alone. \( \bullet \), \text{Vibrio} proteinase; \( \Delta \), subtilisin; \( \text{I} \), thermolysin; \( \text{O} \), V8 or \text{Streptomyces} proteinases. Results with \text{Serratia} 73-kDa proteinase were similar to those with \text{Serratia} 56-kDa proteinase. Note that V8 and \text{Streptomyces} proteinases did not release kallikrein, which indicates that neither Hageman factor nor prekallikrein was activated.

... proteinase (not shown). Two different types of results in the experiments were obtained depending upon proteinases. Namely, anti-Hageman factor antibody inhibited kallikrein generation above 90% by microbial proteinases, except for \( \text{V. vulnificus} \) proteinase, subtilisin, and thermolysin which were inhibited to a lesser extent (>65%, Table I). The same results were obtained by using CTI (Fig. 1, A and B). Therefore, we suggest that kallikrein generation in plasma is mostly dependent on Hageman factor activation in one group (group 1), while in the other group (group 2, e.g. \( \text{V. vulnificus} \) proteinase, subtilisin, and thermolysin) the Hageman factor-dependent generation of kallikrein comprises about 66% (or more) of the total and the rest probably results from direct activation of prekallikrein. In addition, group 3, which includes V8 and \text{Streptomyces} proteinases, did not generate kallikrein activity via this cascade.

To examine the actual occurrence of Hageman factor activation in plasma, the plasma was incubated with the microbial proteinases as described above, and the generation of hydrolytic capacity toward Pro-Phe-Arg-MCA (preferred substrate for activated Hageman factor) was measured. All proteinases examined in groups 1 and 2 generated Pro-Phe-Arg-MCA cleaving activity in a time-dependent manner, but group 3 did not (not shown). The Pro-Phe-Arg-MCA hydrolytic activity was demonstrated to be mainly attributable to the activated Hageman factor generated, because the hydrolytic activity generated was inhibited more than 85% by treatment with anti-Hageman factor antibody (Table I). This result indicates that the proteinases of group 1 and 2 indeed activate Hageman factor in plasma. In the experiments using group 2 proteinases, the anti-Hageman factor antibody blocked Pro-Phe-Arg-MCA cleaving activity about 75-77% (not >85%) in plasma (Table I). The peptide Pro-Phe-Arg-MCA was a 3 times poorer substrate than Z-Phe-Arg-MCA for kallikrein (17). Thus, cleavage of each peptide might have resulted in a slightly different inhibition profile (see Table I, the column of Anti-Hageman factor Ab); for example, 68% (Z-Phe-Arg-MCA) versus 76% (Pro-Phe-Arg-MCA) inhibition for subtilisin. This suggested that this group of proteinases activates prekallikrein and that the kallikrein may have cleaved Pro-Phe-Arg-MCA less efficiently.

Direct Activation of Hageman Factor by Microbial Proteinases in Vitro—To confirm the direct activation of Hageman factor by the microbial proteinases, purified guinea pig Hageman factor was incubated with the microbial proteinases, and the resulting generation of activated Hageman factor was measured by using Pro-Phe-Arg-MCA. The results of the experiment, shown in Fig. 2, demonstrated that all the proteinases except V8 and \text{Streptomyces} proteinases activated Hageman factor in a time-dependent manner, similar to the situation in plasma. The activation of Hageman factor in the presence of EDTA by the 56- and 60-kDa proteinases, \( \text{P. aeruginosa} \) alkaline proteinase and elastase, \( \text{V. vulnificus} \) proteinase and others was examined. The results showed that EDTA completely inhibits these microbial proteinases and no activation of Hageman factor is observed (not shown), which indicates that these metalloproteinases are responsible for the activation of Hageman factor either in plasma or in purified systems but not by contaminating serine proteinases.

Direct Activation of Prekallikrein by Microbial Proteinases in Vitro—As described above, kallikrein generation in plasma by the group 2 proteinases such as \( \text{V. vulnificus} \) proteinase, thermolysin, and subtilisin is inhibited to a lesser extent by CTI and by anti-Hageman factor antibody. This indicates that these proteinases may activate prekallikrein directly in addition to Hageman factor (Fig. 1B and Table I). To examine this possibility, the microbial proteinases were incubated with purified guinea pig prekallikrein, and the resulting kallikrein release was measured by using the synthetic substrate Z-Phe-Arg-MCA. The results, shown in Fig. 3, indicate that \( \text{V. vulnificus} \) proteinase strongly activated prekallikrein, and subtilisin and thermolysin did so moderately, whereas all other proteinases such as those in groups 1 and 3 did not.

Simultaneous Activation of the Hageman Factor-Prekallikrein Cascade by Microbial Proteinases in Vitro—In this series of experiments, both purified guinea pig Hageman factor and prekallikrein were mixed together as in the case of plasma, and the mixture was incubated with microbial pro-
Microbial Proteinases and Kinin Generating System

TABLE I

Vascular permeability enhancing activity of microbial proteinases in guinea pig skin and the effects of CTI, STI, or SQ 20881

Proteinases (micromegs/0.1 ml) alone or mixed with CTI, STI, or SQ 20881 were injected into the skin after intravenous injection of Evans blue into the animals.

<table>
<thead>
<tr>
<th>Microbial proteinase</th>
<th>1 μg proteinase</th>
<th>3 μg proteinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
<td>With CTI</td>
</tr>
<tr>
<td>56-kDa proteinase</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>60-kDa proteinase</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>73-kDa proteinase</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td><em>Pseudomonas</em> alkaline proteinase</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td><em>Pseudomonas</em> elastase</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td><em>Aspergillus</em> proteinase</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>V8 proteinase</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>V8 proteinase</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td><em>Streptomyces</em> proteinase</td>
<td>6</td>
<td>-</td>
</tr>
</tbody>
</table>

*Quantitated spectrophotometrically after extraction of Evans blue dye from the injection sites with formamide for 48 h at 60 °C (25). Three injections were given and the results were expressed as the mean of three determinations.
'Kinin degraded the inhibitor, thus, the locally produced kinin was retained for a longer period without degradation.
Proteolytic activity of the microbial proteinase itself was inhibited by these inhibitors; thus, no effect on dye-leakage by the proteinase was seen.

Proteinases to determine the ability of each proteinase to generate kallikrein. In these experiments, the kallikrein substrate Z-Phe-Arg-MCA was used. The results, shown in Fig. A and B, indicate a great enhancement of Hageman factor-pre-kallikrein activation by all proteinases except V8 and *Streptomyces* proteinases. *P. aeruginosa* elastase generated kallikrein amidolytic activity very slowly in plasma (Fig. 1A). In the purified prekallikrein system, however, *P. aeruginosa* elastase released a larger amount of kallikrein than in plasma (Fig. 4A). This difference may be explained by the fact that *P. aeruginosa* elastase may be inhibited more strongly by plasma a2M than other proteinases.

Enhancement of Vascular Permeability by Microbial Proteinases: Dependence on Hageman Factor-Kallikrein Cascade—All the microbial proteinases examined enhanced vascular permeability in guinea pig skin within a few minutes after intradermal injection (Table II). We then determined whether the vascular permeability reaction caused by the microbial proteinases was dependent on the Hageman factor-pre-kallikrein pathway by simultaneous injection of CTI or STI in guinea pig skin. The results shown in Table II indicate that the permeability enhancement caused by the group 1 proteinases such as 56-, 60-, and 73-kDa proteinases, *P. aeruginosa* proteinase and elastase, and *Aspergillus* proteinase was completely blocked by CTI or STI, as in the *in vitro* experiments. In the case of the group 2 proteinases such as V. vulnificus proteinase, subtilisin, and thermolysin, a marked inhibition by STI was observed, whereas the inhibition by CTI was partial (Table II). These inhibitory patterns with CTI and STI were consistent with those for kallikrein inhibition in plasma, as described above.

In addition, the permeability reaction caused by the proteinases in these groups was augmented by a simultaneous injection of the kinin potentiator SQ 20881 (an inhibitor of kininase II), demonstrating a possible involvement of the chemical mediator bradykinin in the skin as a result of kallikrein generation (Table II).

The group 3 proteinases such as V8 and *Streptomyces* proteinases caused a similar permeability enhancement, but it was weak. This enhancement was also augmented by simultaneous injection of these proteinases with the kinin potentiator SQ 20881 (Table II) and was decreased by injecting carboxypeptidase B, a kinin degrading enzyme (not shown). This indicates that these two proteinases seem to cause vascular permeability by direct release of kinin from kinogen(s) without participation of the Hageman factor or prekallikrein cascade. The permeability activity of the microbial proteinases was not affected by the antihistamine Triprolidine (200 μg/kg) (not shown).

Direct Kinin Generation from High Molecular Weight Kinogen in Vitro—Experiments were also done to examine whether V8 and *Streptomyces* proteinases can liberate kinin directly from high molecular weight kinogen. As shown in Table III, these proteinases released significant amounts of kinin from the kinogen. However, the proteinases were much less efficient than guinea pig plasma kallikrein in releasing kinin. A prolonged incubation of high molecular

**TABLE III**

Generation of kinin from high molecular weight kinogen by microbial proteinases

Quantitated by enzyme-immunoassay (MARKIT® bradykinin kit) using synthetic bradykinin as the standard. All values were after a 10-min incubation with each proteinase.

<table>
<thead>
<tr>
<th>Proteinase used</th>
<th>0.5 μg proteinase</th>
<th>1.0 μg proteinase</th>
<th>10.0 μg proteinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kinin release</td>
<td>ng/ml</td>
<td></td>
</tr>
<tr>
<td>High molecular weight kinogen alone</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>280</td>
<td>480</td>
<td>1600</td>
</tr>
<tr>
<td>V8 proteinase</td>
<td>125</td>
<td>250</td>
<td>224</td>
</tr>
<tr>
<td><em>Streptomyces</em> proteinase</td>
<td>96</td>
<td>224</td>
<td>210</td>
</tr>
</tbody>
</table>

*This value was subtracted as a background.
†This value was obtained with 2 μg of kallikrein/assay mixture.
‡In separate experiments, synthetic bradykinin was found to be degraded by these proteinases as assayed by this method.
weight kininogen with proteinases did not necessarily produce an increased kinin content (not shown). It was found by contrast that at a high dose these proteinases degraded synthetic bradykinin. For example, 0.3 μg of bradykinin was completely degraded by 1.0 μg each of V8 and Streptomyces proteinases within 10 min (not shown).

Concluding Remarks—All microbial proteinases tested caused the vascular permeability reaction in guinea pig skin through the kinin-generating cascade or by direct generation of kinin from high molecular weight kininogen. Based on analyses of each proteolytic cascade step using purified zymogens, specific antibodies and inhibitors, we conclude that the vascular permeability caused by microbial proteinases not only depends on Hageman factor activation but also on pre-kallikrein activation and direct kinin release from kininogens. The different modes of activation by different proteinases may be attributed to the substrate specificity of each proteinase.

The release of kinin thus generated appears to have significant pathological consequences including hemorrhage, edema, and inflammation in seratial, pseudomonal, and other microbial infections, and in some viral infections reported recently (26). This notion is in accord with the fact that kinin generation is highly elevated during pulmonary inflammation in asthmatic subjects (27) and in rhinovirus-infected colds in human subjects where vascular permeability is increased and high amounts of proteinase and kinin are secreted in the nasal discharge (26). Furthermore, Cohn's rule of enhanced kinin generation in bacterial infections is suggested because bacterial proteinases simultaneously and rapidly inactivate plasma proteinase inhibitors (such as α1-proteinase inhibitor, α2-antiplasmin, C1-esterase inhibitor, and antithrombin-III) (28–30) that are known to play a vital role in regulating blood coagulation, the complement system, and the kinin generating cascade.

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