INFECTION AND IMMUNITY, June 1985, p. 747–753
0019-9567/85/060747-07$02.00/0

Vol. 48, No. 3

A Serratial Protease Causes Vascular Permeability Reaction by Activation of the Hageman Factor-Dependent Pathway in Guinea Pigs

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Received 31 December 1984/Accepted 19 February 1985

The 56-kilodalton (56K) protease isolated from a culture filtrate of *Serratia marcescens* caused vascular permeability enhancement followed by edema formation when injected into guinea pig peripheral corneas and subconjunctival space or skin. The character and the mechanism of permeability enhancement were analyzed in vivo. The enhancement was maximum at 5 to 10 min. The permeability reaction increased exponentially by the amount of enzyme used. The enhancement of permeability induced by the 56K protease was not affected by treatment with an antihistamine but was greatly augmented by simultaneous injection of a kinin potentiator, Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro-OH (SQ 20,881). Furthermore, the permeability activity of the protease, but not the amidolytic activity, was inhibited by soybean trypsin inhibitor, a well-known inhibitor of plasma kallikrein, as well as by corn trypsin inhibitor, the best inhibitor of activated Hageman factor. Results of these in vivo studies indicate that the permeability-enhancing reaction induced by the 56K protease is caused by activation of the Hageman factor-dependent pathway in the tissue. The permeability-increasing activity of the 56K protease was parallel with the enzyme activity. Serratial lipopolysaccharide did not produce a permeability enhancement reaction within 30 min when injected into guinea pig skin. These results are consistent with the results of recent in vitro experiments in which activation of the purified Hageman factor but not of prekallikrein by the 56K protease was elucidated (Matsumoto et al., J. Biochem. (Tokyo) 96:739–749, 1984). Thus, the molecular mechanism described above appears to be operative in the pathogenesis of corneal edema and chemosis, which is induced by *S. marcescens*, in addition to the direct tissue destruction by the protease.

In recent decades, the significance of opportunistic infections with multiple drug resistance has been realized. *Pseudomonas* species, *Escherichia coli*, and *Serratia* species are three major agents among the gram-negative bacilli that are responsible for this. The molecular mechanisms of pathogenesis are fairly well clarified in *Pseudomonas* species and *E. coli* in view of specific protein exotoxins (5, 7, 8, 18). There have been no reports on the specific exotoxins in the pathogenesis of *Serratia* infection, comparable to those of *Pseudomonas* species or *E. coli*. We have been investigating the pathogenesis of ocular infections with *Serratia* species, focusing on the proteases secreted by this bacteria, since 1980 (15). Very recently, Lyerly and Kreger have reported the critical role that this protease plays in the pathogenesis of serratial pneumonia (14).

In the field of ophthalmology, *Serratia* keratitis with corneal ulcer is one of the most prevalent (1, 3, 4, 11, 20, 21, 23). We have separated four distinct proteases: two metalloproteases of 56 and 60 kilodaltons (56K and 60K, respectively) and two thiol proteases, 73Ka and 73Kb, to homogeneity from a culture supernatant of *Serratia marcescens* kums 3958 isolated from the corneal lesion of a patient (16). In addition, we experimentally produced corneal damage and ulceration in rabbits by injecting the 56K protease into the center of the cornea (15). Independently, Lyerly and Kreger have reported a purified serratial metalloprotease but with microheterogeneity (13) and have shown that the protease(s) caused liquefactive necrosis in rabbit corneas with destruction of proteoglycan substrate rather than collagen fibrils (12). Thus, the direct destruction of substances in the corneal tissue by serratial extracellular proteases was found to be one of the major causes of ulcer formation in serratial keratitis.

However, the intracorneal edema at the peripheral area and chemosis, another remarkable aspect in the keratitis, remain to be elucidated. In our preliminary study with guinea pigs, an injection of the 56K protease into the periphery of the cornea or into the conjunctiva caused vascular permeability enhancement and mimicked, at least in part, the corneal edema and the chemosis. Therefore, it is necessary to investigate the permeability enhancement reaction induced by the 56K protease to realize the mechanism of corneal edema and chemosis in serratial keratitis.

Conroy et al. previously described vascular permeability-increasing activity of *S. marcescens* protease(s) in rat skin (2). They have reported that the cause of the reaction is unclear, because the reaction is not affected by treatment with antihistamines or antiserotonines, or by depletion of complement with cobra venom factor and polymorphonuclear leukocytes with specific antibody in rats (2). Also, during attempts to characterize the 56K protease, we found that its substrate specificity for synthetic peptides was almost identical to that of plasma kallikrein, which is a well-known Hageman factor activator, and we verified that Hageman factor in guinea pigs indeed was activated by the 56K protease in vitro (17). As reported previously, the activated Hageman factor is a potent permeability factor (24, 25), and the activity is caused by kallikrein generation followed by kinin formation in vivo (24). In view of these observations, we investigated whether the 56K protease

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causes an increase in permeability by activating the Hageman factor and the subsequent kinin-generating cascade reaction using guinea pigs as a model.

MATERIALS AND METHODS

Animals and reagents. Albino Hartley guinea pigs of both sexes, (weight, 400 to 600 g) were used. Evans blue and histamine were obtained from Wako Pure Chemical Co., Osaka, Japan. Soybean trypsin inhibitor (SBTI), carboxypeptidase B (CPB), and *S. marcescens* lipopolysaccharide were purchased from Sigma Chemical Co., St. Louis, Mo. Triprolidine hydrochloride was a product of Tanabe Seiyaku Co. Ltd., Osaka, Japan. Synthetic bradykinin was purchased from Sandoz Pharmaceuticals, Hanover, N.J. Serratial 56K protease was purified from the culture supernatant of *S. marcescens* kums 3958 as described previously (16). Corn trypsin inhibitor (CTI), which was prepared as described previously (6), was kindly provided by Yoshio Hojima of Scripps Clinic, La Jolla, Calif. Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro-OH (SQ 20,881) was a gift of E. R. & Sons, Squibb Princeton, N.J. 2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid (carboxypeptidase N inhibitor; CPNI) was obtained from Calbiochem-Behring, La Jolla, Calif.

Vascular permeability enhancement reaction in guinea pig eyes. The 56K protease (1 μg/0.1 ml of saline) was injected into the peripheral area of the cornea or the subconjunctival space near the limbus in the right eye immediately after Evans blue (30 mg/kg) was injected intravenously into the guinea pigs. A similar volume of 0.9% saline was injected into the left eye as a control. The injection sites were observed carefully with a biomicroscope for any dye leakage.

Measurement of permeability activity. The permeability assay was usually performed with guinea pig skin. The 56K protease, serratial lipopolysaccharide, histamine, and bradykinin were diluted, to various concentrations with 0.9% saline. A 0.1-ml fraction of the sample was injected intradermally into the clipped flank of a guinea pig immediately after an intravenous injection of 30 mg of Evans blue (2.5% solution in 0.9% saline) per kg. The permeability activity of the sample was quantitated by measuring the extravasated Evans blue in the guinea pig skin by the dye extraction method of Udaka et al. (22) as described previously (24).

To investigate the pharmacological effects of reagents to the permeability reaction induced by 56K protease, two types of treatments, local and systemic, were performed. In the case of local treatment, each of SQ 20,881, CPNI, CPB, SBTI, or CTI was mixed with 56K protease and injected into guinea pig skin to measure the augmented or reduced permeability reaction. For each assay a standard curve based on three or four different doses of 56K protease was obtained in which a linear relationship between the dye leakage and the logarithm of the concentration of 56K protease exists. We then quantified the extent of augmentation or inhibition of permeability activity of 56K protease. In the case of systemic treatment with an antihistamine, animals were injected intravenously with triprolidine (200 μg/kg) 90 min before the intradermal injection of the samples, such as 56K protease and histamine. Histamine was used to confirm the potency of the antihistamine treatment. In the case of treatment with CPNI, animals were injected intravenously with 30 mg of CPNI in 1 ml of phosphate-buffered saline 30 min before the intradermal injection of the samples, such as 56K protease, bradykinin, and histamine. Histamine was used to examine the positive responsiveness of the individual guinea pigs used.

To study the time course of the permeability reaction induced by the 56K protease, two doses (3 and 10 μg) were injected at 12, 6, and 3 h and 30, 20, 10, 5, and 0 min before intravenous injection of Evans blue into the skin of two guinea pigs. At 30 min after the injection of dye, the animals were killed by exsanguination under ether anesthesia, and the permeability reaction in the harvested skin was quantitated as described above. The time course of the permeability reaction induced by serratial lipopolysaccharide (0.1, 1.0, and 10 μg in 0.1 ml of saline) was examined similarly for a period of 48 h.

Measurement of enzymatic activity of 56K protease. The enzyme activity of 56K protease was measured as described

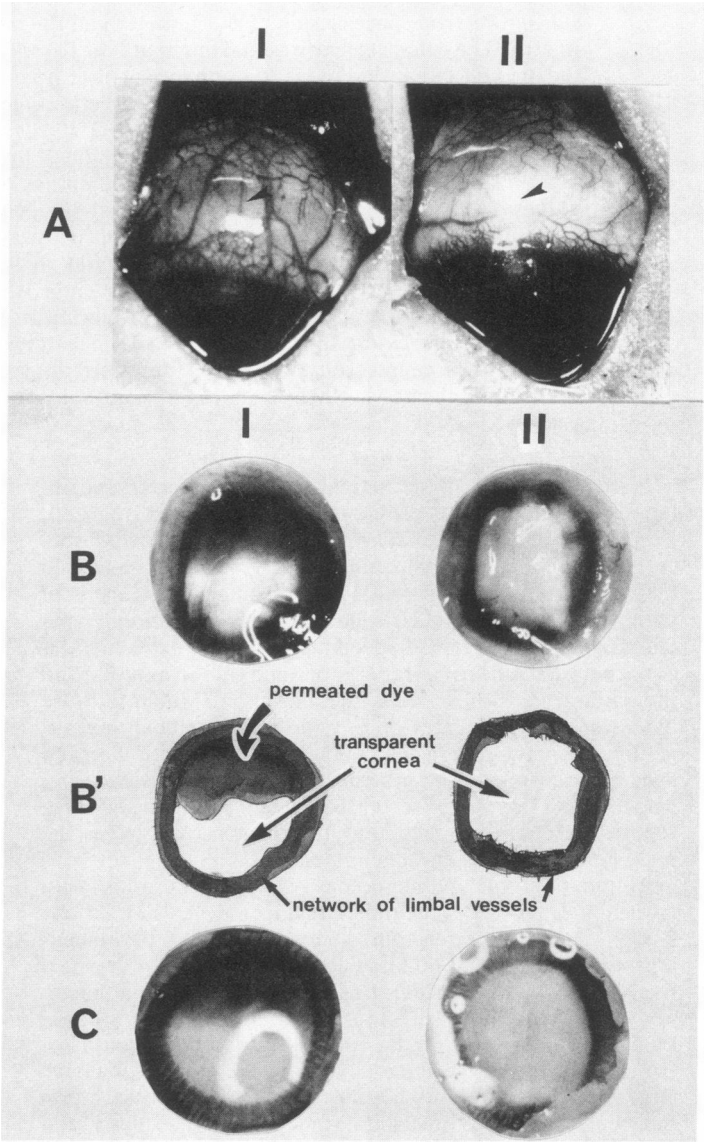

FIG. 1. Vascular permeability in guinea pig eyes 30 min after injection of serratial 56K protease in the upper conjunctiva and upper peripheral cornea. (A) Conjunctiva; arrowheads indicate injection sites. (B) Outer view of excised cornea. (B') Illustrations of (B). (C) Inner view of the cornea shown in (B). (I) 1.0 μg of the protease. (II) Saline (control), both of these photographs were obtained from the same guinea pig.

previously with gelatin (16) and Z-Phe-Arg-MCA (4-methylcoumaryl-7-amide) (17) as the substrates. To examine the effect of the reagents used in the permeability assay to the enzyme activity of 56K protease, 56K protease was preincubated with each reagent in phosphate-buffered saline (pH 7.4) for 20 min at room temperature, and the remaining activity was measured.

RESULTS

Vascular permeability enhancement reaction in guinea pig eyes. When 1 μg of the 56K protease (in 5 μl of saline) was injected into the peripheral cornea and subconjunctival space of guinea pigs, intense extravascular leakage of the dye was observed at each injection site (Fig. 1). The dye began to leak from limbal or conjunctival vessels 1 to 3 min after injection of the protease and was gradually accumulated in the injection site of the peripheral cornea and the subconjunctiva (Fig. 1). However, when same dose of protease was injected into the central cornea of the guinea pigs, extravascular leakage of the dye was not observed in the cornea from any direction of the limbus but was observed somewhat in the anterior chamber from the iris surface (data not shown).

Analysis of permeability enhancement activity of the 56K protease in guinea pig skin. The 56K protease caused a permeability response in guinea pig skin. However, the heat-treated 56K protease (65°C for 20 min) showed much less of a permeability response because of the inactivation of the 56K protease (Fig. 2). On the other hand, serratial lipopolysaccharide (0.1, 1, and 10 μg) showed little permeability activity within 30 min after sample injection (Fig. 2).

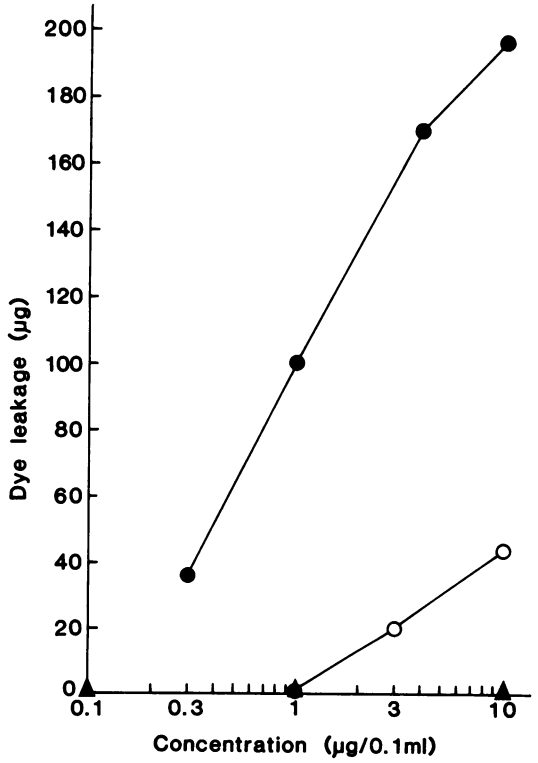

FIG. 2. Effect of heat treatment on the permeability activity of the 56K protease and the effect of serratial lipopolysaccharide permeability 30 min after injection. Symbols: ●, 56K protease; ○, heat-treated 56K protease; ▲, serratial lipopolysaccharide.

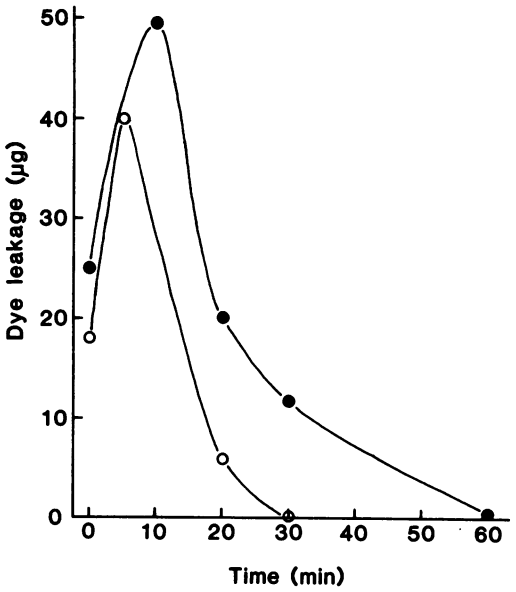

FIG. 3. Time course of permeability of the 56K protease. Serratial 56K protease at concentrations of 3 μg (○) and 10 μg (●) was used.

However, it showed weak permeability activity with a 10-μg dose only at 60 min after injection during a 48-h observation period (data not shown). When 10 μg of the 56K protease was injected, the most intense extravascular leakage of dye was observed at 10 min after intradermal injection of the enzyme, but the effect diminished to almost undetectable levels in 60 min or longer (Fig. 3). When 3 μg of the enzyme was used, maximum leakage of the dye was observed at 5 min, and it became undetectable at 30 min after injection (Fig. 3). This confirms that the vascular permeability-enhancing factor has a short half-life and acts locally.

Doses of the 56K protease and bradykinin and their permeabilities in vivo. A linear relationship between the logarithm of the injected dose and the amount of extravascular dye was observed for the dosage range from 0.3 to 10 μg for the 56K protease or bradykinin (Fig. 4). Bradykinin exhibited a small linear increment in the range from 0.1 to 10 μg. The increase in the permeability activity of the 56K protease was about 2.4-fold greater than that of bradykinin on a weight basis (at 10 μg; Fig. 4).

Effect of antihistamine on the permeability caused by the 56K protease. The permeability activity of the 56K protease was not affected by treatment with the antihistamine triprolidine, whereas that induced by histamine was completely inhibited as expected (Fig. 5). These results indicate that the permeability activity of the 56K protease does not involve release of histamine.

Effect of inhibition of bradykinin-destroying enzyme by SQ 20,881 and CPNI in vivo. SQ 20,881 is known to potentiate kinin activity by inhibiting kinin-converting enzyme (angiotensin-converting enzyme). The permeability activity of the 56K protease (0.3 μg) was not affected by mixing with SQ 20,881 (50 μM), but that of the higher dose of the enzyme (1 μg) was greatly increased (twofold) by mixing with SQ 20,881 as compared with the control (1 μg of 56K protease alone; Fig. 6B). The permeability activities of 1 μg of the 56K protease and bradykinin were also increased by CPNI to 136 and 200%, respectively, even when given intravenously (data not shown). When 3 μg of the 56K protease and

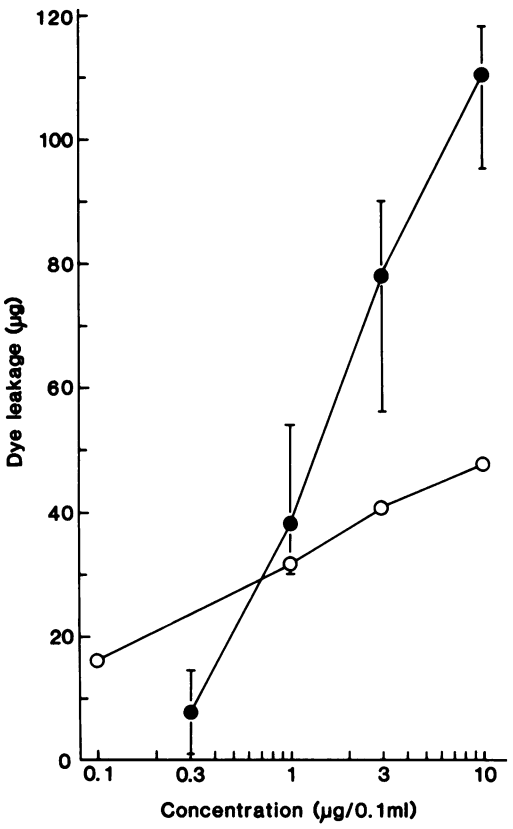

FIG. 4. Comparison of permeability of the 56K protease and bradykinin. 56K protease (●) in triplicate experiments and bradykinin (○) are shown.

3 μg of bradykinin were mixed with CPNI (3.3 mg), their permeability activities were increased by 160 and 196%, respectively (Fig. 6D).

Effects of CPB (kininase), SBTI, and CTI on the permeability of the 56K protease in vivo. Neither 8.7 nor 1.8 U of CPB inhibited the permeability activity of 56K protease at any dose (data not shown). We confirmed that the 56K protease did not affect the activity of CPB (data not shown). The permeability activity of the 56K protease (10 μg) was inhibited about 90% by mixing with SBTI (5 nmol). The extent of this inhibition became more remarkable when a greater amount of inhibitor than enzyme was used (Fig. 7.) Similarly, a marked inhibition was observed by mixing the 56K protease with CTI (2 nmol). This inhibition was also more pronounced when the smaller dose of the enzyme was used (Fig. 8). These results suggest the involvement of both activated Hageman factor (CTI) and kallikrein (SBTI) in this cascade system (Fig. 9).

DISCUSSION

The 56K protease caused a short-lasting permeability enhancement reaction when injected into guinea pig skin. Histamine and kinin are well known as mediators of the short-lasting permeability reaction, and the release of histamine from mast cells, such as an immunoglobulin E-mediated reaction or a complement-dependent reaction (19) and release of bradykinin by the Hageman factor-kallikrein-kinin cascade, recently have been well investigated in guinea pig skin. Because the permeability reaction induced by the 56K protease was resistant to treatment with antihistamine (Fig. 5), histamine release is not the cause. Conroy et al. have produced a similar result in rats (2).

The Hageman factor-kallikrein-kinin cascade consists of

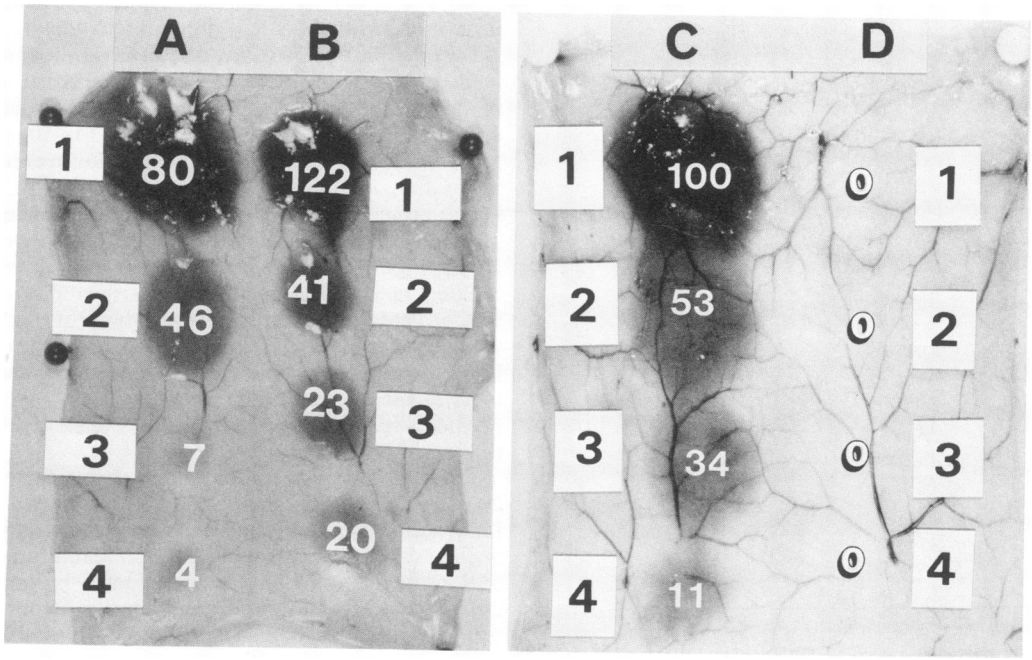

FIG. 5. Inhibition of the permeability induced by the 56K protease and histamine by antihistamine. In (A and B) there was no pretreatment with antihistamine. (A) 56K protease at 10- (1), 3.0- (2), 1.0- (3), and 0.3-μg (4) dose per injection. (B) Histamine at 3.0- (1), 1.0- (2), 0.3- (3), and 0.1-μg (4) dose per injection, respectively. (C and D) Results after pretreatment with antihistamine (200 μg/kg). (C) The same doses of 56K protease as in (A). (D) The same doses of histamine as in (B). (A and B) and (C and D) were obtained in the same animal, respectively. The amount (in micrograms) of dye leakage quantified after photography is shown with white letters.

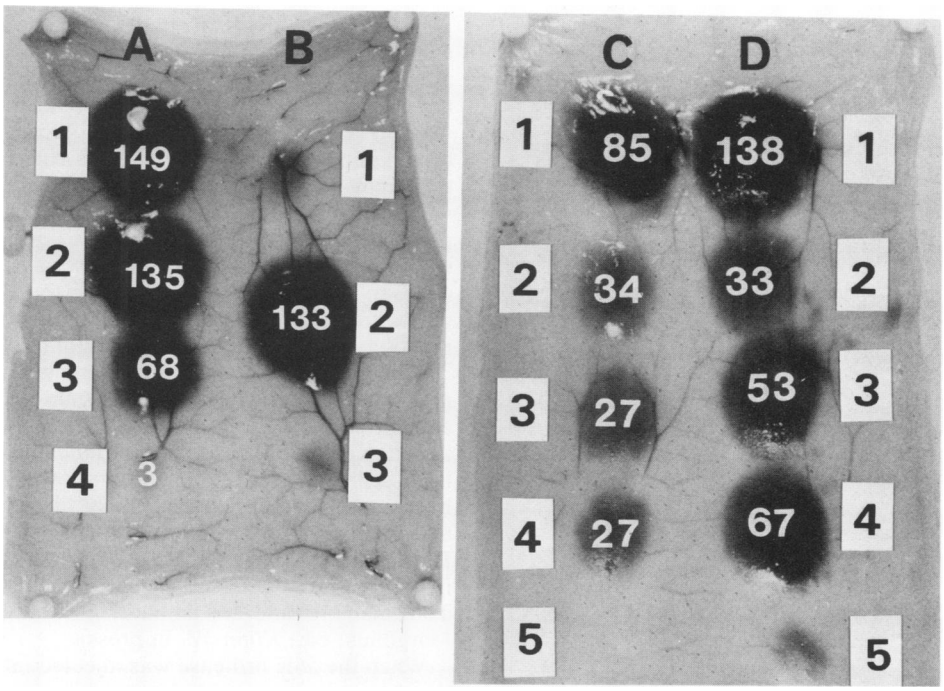

FIG. 6. Effect of SQ 20,881 and CPNI on the permeability of the 56K protease. (A) 56K protease at 10- (1), 3.0- (2), 1.0- (3), and 0.3-μg (4). (B) SQ 20,881 (5 nmol) alone (1) or that with 1.0 μg (2) and 0.3 μg (3) of the 56K protease. (C) 3.0 μg (1) and 1.0 μg (2) of the 56K protease alone; 3.0 μg (3) and 1.0 μg (4) of bradykinin alone; saline (5). (D) 3.0 μg (1) and 1.0 μg (2) of the 56K protease mixed with CPNI (3.3 mg); 3.0 μg (3) and 1.0 μg (4) of bradykinin mixed with CPNI (3.3 mg); CPNI (3.3 mg) alone (5). Numbers in white letters show the amount (in micrograms) of dye leakage.

the generation of kallikrein from prekallikrein by activated Hageman factor and the release of bradykinin from high-molecular-weight kininogen by the kallikrein that was generated. The bradykinin thus released is quickly inactivated by the kinin-destroying enzymes (angiotensin-converting enzyme) and carboxypeptidase N (CPN). The permeability reaction mediated by this cascade therefore should be affected by pharmacological reagents if they have any effects on any one step of the cascade (unpublished data).

As shown in the activation scheme (Fig. 9), bradykinin is degraded by two separate enzymes, ACE and CPN, both of which can be specifically inhibited by SQ 20,881 and CPNI, respectively. Figures 6B and D provide evidence that both SQ 20,881 and CPNI are effective in augmenting the permeability-enhancing activity of the 56K protease compared with the results obtained during the absence of these inhibitors. This again confirms that the permeability caused by the 56K protease is mediated through bradykinin formation. The kinin-generating cascade in situ can be envisaged, but no evidence of a direct kinin-generating system was confirmed by the negative results in the experiments with SBTI and CTI (Fig. 7 and 8).

SBTI and CTI are well-established, specific inhibitors for kallikrein and activated Hageman factor, respectively, but they have no effect on the protease activity of the 56K protease. These inhibitors suppressed the permeability enhancement induced by the 56K protease (Fig. 7 and 8); thus, it is suggested that the involvement of kallikrein and activated Hageman factor cause the permeability response in this cascade (Fig. 9). The heat-treated enzyme lost about 97% of its proteolytic activity in vitro (16), and this paralleled its loss in permeability-enhancing activity (Fig. 2).

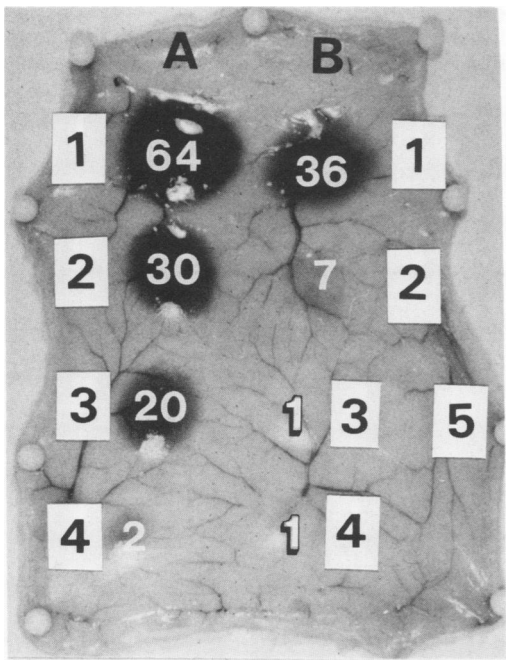

FIG. 7. Effect of SBTI on the permeability of the 56K protease. (A) 56K protease at 10 (1), 3.0 (2), 1.0 (3), and 0.3 μg (4). (B) 10 (1), 3.0 (2), 1.0 (3), and 0.3 μg (4) of the 56K protease mixed with SBTI (5 nmol); SBTI (5 nmol) alone (5). Numbers in white letters show the amount (in micrograms) of dye leakage.

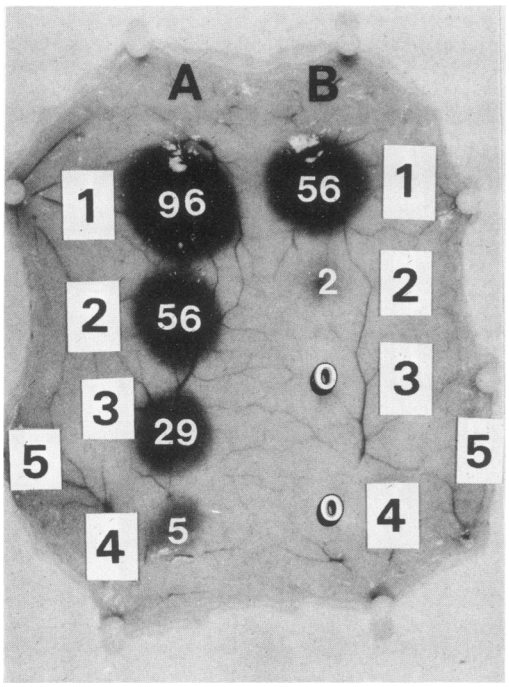

FIG. 8. Effect of CTI on the permeability of the 56K protease. (A) 56K protease at 10 (1), 3.0 (2), 1.0 (3), and 0.3 μg (4) and saline (5). (B) 56K protease at 10 (1), 3.0 (2), 1.0 (3), and 0.3 μg (4) mixed with CTI (2 nmol) and CTI (2 nmol) alone (5). Numbers in white letters show the amount (in micrograms) of dye leakage.

When another cellular component, lipopolysaccharide, from *S. marcescens* was tested for vascular permeability-enhancing activity in the same assay system, it did not show any activity within 30 min after injection (Fig. 2), negating the possibility that the permeability reaction might be due to the contaminated lipopolysaccharide. Therefore, the permeability reaction induced by the 56K protease is caused by proteolytic activity of the enzyme. These results are consistent with our previous findings in vitro that the 56K protease activates Hageman factor and that the activated Hageman factor then activates prekallikrein. This step may be inhibited by CTI (17).

As described above, from the duration of the permeability enhancement reaction, with the maximum occurring at 5 min after intradermal injection (Fig. 3), the reaction induced by the 56K protease was classified as short lived. However, it was not as short as other reactions of this type. For example, bradykinin and kallikrein in plasma cause a maximum reaction immediately after intradermal injection, and the activated Hageman factor does this in 2 min (9, 24). The presence of this lag phase in the permeability-increasing response caused by the 56K protease suggests that a cascade reaction in situ causes the response. These different lag periods are consistent with the sequence of the cascade reaction of the Hageman factor-kallikrein-kinin system.

Thus, the activation of the Hageman factor-kallikrein-kinin cascade in the interstitial tissue was assumed to be capable of causing the vascular permeability reaction after injection of the 56K protease into the interstitial extravascular tissue of the skin. This assumption is strongly supported by a recent immunohistochemical observation of the wide distribution of the components of the pathway, such as Hageman factor and high-molecular-weight kininogen in the interstitial extravascular tissue in guinea pig skin (J. Tsurata, T. Yamamoto, and T. Kambara, *in* L. M. Greenbaum and H. Margolius, ed., *Kinin IV*, in press).

When the 56K protease was injected into the periphery of the cornea or the subconjunctiva, it caused a permeability enhancement reaction in the injection sites, resulting in corneal swelling and chemosis. This phenomenon may be also attributed to activation of the Hageman factor-kallikrein-kinin system in the vascular tissue near the cornea, such as the limbus, the conjunctiva, and the episclera. This evidence reveals, at least in part, the pathogenesis of edema formation in the cornea and of chemosis by the 56K protease in experimental *S. marcescens* keratitis.

The dose-response curve of the 56K protease causing permeability enhancement was much steeper than that of bradykinin. Therefore, this indicates that the cascade system appears to function as an amplification system of the permeability reaction. The following hypothesis tempts us to investigate the role of the permeability enhancement reaction in keratitis. It is an interesting observation that little tissue destruction but an increase in the permeability of vascular tissue occur frequently in guinea pig eyes in clinical or experimental keratitis; this might be attributed to the

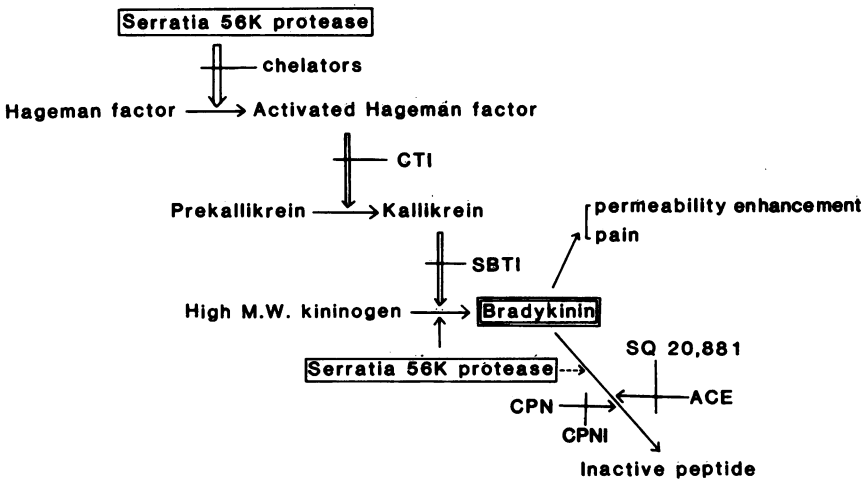

FIG. 9. Activation scheme of the Hageman factor cascade by serratial 56K protease.

inhibition of the tissue destructive protease, 56K protease, by a plasma protease inhibitor, α_2-macroglobulin or guinea pig macroalbumin, which accumulate in the inflammatory site because of the increased permeability. This hypothesis is supported by the observation that the 56K protease was inhibited by human α_2-macroglobulin (10) and by guinea pig macroalbumin (Matsumoto et al., unpublished data). It has been reported that there is a 10-fold increase in the concentration of guinea pig macroalbumin in skin tissue when the permeability is increased by injecting activated Hageman factor (10). For further investigation of this hypothesis, several experiments based on the findings of this study will be required; for example, to determine whether the 56K protease simultaneously injected with SBTI or CTI, which block the permeability enhancement without inhibiting the protease activity of the 56K protease, induces more intense tissue destruction of the injected area. Such investigations are now in progress in our laboratories.

A possible involvement of the mechanisms described above in the pathogenesis of serratial pneumonia (14) is also an interesting aspect of the results of this study.

ACKNOWLEDGMENTS

We thank R. Okamura and T. Kambara for interest and discussions.

This study was supported in part by Grants in Aid for Research from the Ministry of Education, Science, and Culture of Japan to T.Y. and H.M.

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