A High-Molecular-Weight Trypsinlike Protease in the Skin Sites of Delayed Hypersensitivity in Guinea Pigs

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A trypsinlike protease was extracted from the delayed hypersensitivity skin sites in guinea pigs. Extractable amounts of the enzyme were chronologically paralleled with the gross appearance of the inflammation, and the maximum activity from the inflamed sites at 24–36 hours was about 20 times stronger than that from normal skin, suggesting a potential role in the pathogenesis of the delayed hypersensitivity reaction. The enzyme, which suitably hydrolyzed t-butyloxycarbonyl-phenylalanyl-seryl-arginine 4-methylcoumaryl-7-amide, was partially purified by isoelectric focusing or by gel filtration. The enzyme demonstrated a single peak of activity on the former column with an apparent isoelectric point of 4.2, and in the latter it showed an apparent molecular weight of 600,000 (600K-protease). When incubated with ^{3}H-diisopropylfluorophosphate, the enzyme lost all amidolytic activity and yielded a single band of radioactivity in polyacrylamide disk gel electrophoresis in the presence of sodium dodecyl sulfate, and a single peak of radioactivity in gel filtration, both having an apparent molecular weight of 31,000–33,000 (31K-protease). That the 600K-protease might be a complex with α_2-macroglobulin was ruled out. The 31K-protease was separated from the 600K-protease by gel filtration in the presence of 6 M guanidine hydrochloride, and was renatured to an active form. An apparent isoelectric point of the 31K-protease observed was 9.4, suggesting that the 600K-protease may be a complex of 31K-protease with an acidic carrier molecule(s). Both proteases, 31K- and 600K-protease, had identical substrate specificity, a pH profile of amidolytic activity, and susceptibility to exogenous protease inhibitors. However, when sensitivities to intrinsic protease inhibitors in guinea pig plasma, two kinds of trypsin inhibitor, and α_2-macroglobulin were compared, the 600K-protease was at least 100 times more resistant than the 31K-protease. It was supposed that one of the pathophysiologically significant functions of the complex formation might be to maintain the enzyme activity longer *in vivo*. (Am J Pathol 1984, 114:250–263)

THE PATHOGENESIS of the delayed hypersensitivity reaction (DHR) is the focus of much current interest. Histologically, the accumulation of mononuclear cells in the reaction sites is one of the most characteristic features in DHR, and to clarify their role would be primary importance to understand the inflammatory process of DHR. One of the authors (T.K.) has reported the presence of at least three macrophage-chemotactic factors at DHR skin sites (MCFS) in the guinea pigs and proposed their participation in mononuclear cell accumulation in the lesions.[1–4] Because the strongest one (MCFS-1) was revealed to be a protein with an apparent molecular weight of 150,000, and other protein molecules were also proposed as chemical mediators of DHR,[5] it became of interest to investigate protease as a regulating factor of chemical mediators with a protein nature. We began by finding a protease(s) in the skin extract.[6] In this study we focused on the protease(s) whose activity changed with the chronologic relationship to pathologic event(s) in DHR, and newly developed fluorogenic peptide substrates which have high specificity and sensitivity were used. In the course of these studies, we encountered a large protease with an apparent molecular weight of 600,000 in the extract

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of DHR sites, and changes of the protease activity in the extracts of DHR sites corresponded with the time course of the gross appearance of the inflammation. The results were sufficient to lead us to postulate its potential participation in the development of DHR or in the regulation of the reaction. We analyzed the enzymatic characteristics of this unique protease first.

Materials and Methods

Animals

Female Albino-Hartley strain guinea pigs weighing 300–400 g were fed a semisynthesized laboratory diet (G Standard, Nippon Nosan, Yokohama, Japan) and water *ad libitum* during the study.

Chemicals

Bovine γ-globulin (BGG) was obtained from Armour (Kankakee, Ill). Bacto-adjuvant, complete, was obtained from Difco (Detroit, Mich). Hemoglobin, diisopropylfluorophosphate, *p*-chloromercuribenzoate, phenylmethylsulfonylfluoride, N-α-*p*-tosyl-L-lysine-chloromethylketone-HCl, soybean trypsin inhibitor, N-ethylmaleimide, ethylenediaminetetraacetic acid, ovomucoid trypsin inhibitor, benzamidine, blue dextran, thyroglobulin, catalase, ribonuclease A, and bovine pancreatic trypsin were obtained from Sigma (St. Louis, Mo). Trasylol was a gift from Bayer (Osaka, Japan). Fluorogenic peptide substrates, t-butyloxycarbonyl-phenylalanyl-seryl-arginine 4-methylcoumaryl-7-amide (Boc-Phe-Ser-Arg-MCA), carbobenzoxy-phenylalanyl-arginine 4-methylcoumaryl-7-amide (Z-Phe-Arg-MCA), prolyl-phenylalanyl-arginine 4-methylcoumaryl-7-amide (Pro-Phe-Arg-MCA), t-butyloxycarbonyl-valyl-leucyl-lysine 4-methylcoumaryl-7-amide (Boc-Val-Leu-Lys-MCA), benzoyl-arginine 4-methylcoumaryl-7-amide (Bz-Arg-MCA), and aminomethylcoumarine (AMC) were purchased from Protein Research Foundation (Osaka, Japan). Sepharose 6B and Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden). Carrier ampholytes were products of LKB-aminkemi (Broma, Sweden). ^{3}H-acetic anhydride and ^{3}H-diisopropylfluorophosphate were obtained from the Radiochemical Centre, Amersham, United Kingdom. Guanidine hydrochloride and all other chemicals were purchased from Wako Pure Chemicals (Osaka) or Nakarai Chemicals (Kyoto, Japan).

Induction of Delayed Hypersensitivity Reactions

Guinea pigs were immunized to BGG (10 μg per animal) in Freund's complete adjuvant as described previously.[1] Eight days later, 10 μg of BGG in 0.1 ml saline per site was administered intradermally on the clipped flank at 20 sites. Inflamed skin at various ages of DHR from 0 to 72 hours were measured for the mean lesion diameter of redness and the double thickness. DHR developed with maximal intensity of erythema and induration about 19–24 hours after the injection. Precipitating serum antibody to BGG could not be demonstrated by the precipitation test in any of the animals at this time.

Preparation for Skin Extract

Inflamed skin sites were extirpated immediately after the animals had been sacrificed by exsanguination under ether anesthesia. The physiologic saline-treated immunized animals and BGG-treated sites in the normal animals were also extracted as a control. After freezing at −80 C, the pieces of frozen skin were minced with a meat chopper and divided into two parts. One was dehydrated with three changes or 10 volumes of cold acetone as described previously.[7] The skin acetone powders from DHR skin sites of various ages were extracted separately with 10 volumes of 67 mM phosphate buffer (pH 7.4) for 4 hours at 4 C. The clear supernatants were recovered by centrifugation for 20 minutes at 15,000 rpm at 4 C and used as the skin extracts. The other part was directly extracted with 2.5 volumes of the same buffer for 4 hours at 4 C. The extracts obtained were used as a control for the acetone treatment. The volumes of the recovered extracts from the acetone-treated and non-acetone-treated skin were almost the same.

Protein Concentration

The protein concentration of the extracts and fractions from column chromatography was determined from the absorbance at 280 nm (A_{280}), assuming $E^{1\%}_{280} = 10$.

Preparation of Guinea Pig α_2-Macroglobulin and Anti-Guinea Pig α_2-Macroglobulin Rabbit Antibody

Alpha$_2$-Macroglobulin was purified from guinea pig plasma by successive column chromatography using DEAE-Sephadex, lysine-Sepharose 4B, DEAE-Sephadex again, CM-Sephadex, and Sepharose 6B in that order.[8] The apparent molecular weight of the α_2-macroglobulin was 720,000 by gel filtration and 190,000 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in the presence of a reducing agent. The α_2-macroglobulin inhibited caseino-

lytic activity of bovine trypsin but not an amidolytic activity of the same enzyme on Boc-Phe-Ser-Arg-MCA (see below), findings similar to those of reports on the human molecule.

Anti-guinea pig α_2-macroglobulin antibody was raised in rabbits. IgG antibody fraction was purified from the rabbit antisera by ammonium sulfate precipitation, CM-Sephadex column chromatography, DEAE-Sephadex column chromatography, and reprecipitation with ammonium sulfate. The IgG fraction was treated with diisopropylfluorophosphate (final concentration, 1 mM) to block the trace amount of protease contamination in the fraction, dialyzed against phosphate-buffered saline (PBS) containing 5% glucose (pH 7.4), and used as an anti-α_2-macroglobulin antibody. Normal IgG was prepared from rabbit sera in the same way.

Preparation of Trypsin-α_2-Macroglobulin Complex

Fifty microliters of guinea pig α_2-macroglobulin (37.5 μg/ml) were incubated with an equal volume of bovine trypsin (100 μg/ml) for 30 minutes at 37 C in PBS (pH 7.4) and further treated with soybean trypsin inhibitor (final concentration, 100 μM) for 10 minutes at 37 C for the inhibition of unreacted free trypsin molecules. The amidolytic activity of the complex on Boc-Phe-Ser-Arg-MCA was estimated, and the complex was used as a positive control for the immunoprecipitation study after adjustment of the amidolytic activity to a concentration similar to that of the skin protease.

Preparation of Guinea Pig Plasma Trypsin Inhibitors

Two types of trypsin inhibitors were partially purified from guinea pig plasma according to the method of Kobayashi and Nagasawa[9] with the use of DEAE-Sephadex column chromatography with linear salt gradient elution. These inhibitors were separated from α_2-macroglobulin during the column chromatography.

Determination of Protease Activities

The protease activities were measured by amidolytic activities with the use of synthetic fluorogenic substrates and ^{3}H-acetyl hemoglobin. The amidolytic activities were measured by an end-point assay system with synthetic fluorogenic substrates for trypsin-type proteases, Boc-Phe-Ser-Arg-MCA, Z-Phe-Arg-MCA, Pro-Phe-Arg-MCA, Boc-Val-Leu-Lys-MCA, and Bz-Arg-MCA. For the assay, 10 μl of substrate stock solution (5 mM) in dimethyl sulfoxide (DMSO) or water were mixed with 440 μl of assay buffer (final concentration of substrate during reaction, 100 μM) and preincubated for 5 minute at 37 C. Then 50 μl of enzyme solution was added and incubated for 10 minutes at 37 C, and the reaction was terminated by the addition of 20% acetic acid. A concentration of 50 mM Tris-HCl buffer (pH 8.5 or 10) was usually used as the assay buffer, and 200 mM acetate buffer (pH 3–6), 100 mM phosphate buffer (pH 6–8), 200 mM Tris-HCl buffer (pH 8–11), and 200 mM sodium phosphate–sodium hydroxide buffer (pH 11–12) were used for studying the pH dependency of the enzymatic activities. The amount of AMC released by the amidolytic activity was fluorometrically measured with a fluorescence spectrophotometer (Model RF-502, Shimadzu, Kyoto, Japan) with excitation at 380 nm and emission at 440 nm according to Morita et al[10–12] as described previously.[13] In this assay method the enzyme solutions used gave linear amidolytic rates for at least 30 minutes at 37 C. The amidolytic activity was expressed in AMC concentration released per minute.

For the proteolytic assay, ^{3}H-acetyl hemoglobin was prepared by acetylation with ^{3}H-acetic anhydride by the method of Hille et al[14] as described previously.[6] ^{3}H-acetyl hemoglobin (0.75 mg/ml) had a specific activity of 225 cpm/pmol protein, assuming a molecular weight of 69,000. For determination of protease activity, mixtures containing 50 μl of ^{3}H-acetyl hemoglobin (18.75 μg, 65,000 cpm), 50 μl Tris-HCl buffer (pH 8.5), and 50 μl sample were incubated for 60 minutes at 37 C in a shaker. Then 100 μl of nontritiated 2.5% hemoglobin was added, followed by 100 μl of 15% trichloroacetic acid. The radioactivity of 200 μl acid-soluble materials were determined by a Packard Tri-Carb scintillation spectrometer (Model 3385, Packard Instrument Co., Downers Grove, Ill). Appropriate blanks consisting of 67 mM phosphate buffer (pH 7.4) were incubated simultaneously. All assays were conducted in duplicate.

For the inhibition study of amidolytic and proteolytic activity by protease inhibitors, various trypsin inhibitors were added to the partially purified enzyme solutions at a final concentration of 1 mM of the exogenous inhibitors or at arbitrary concentrations of plasma trypsin inhibitors and incubated for 30 minutes at 37 C prior to the enzyme assays. In the case of guinea pig α_2-macroglobulin, soybean trypsin inhibitor was added to the protease-inhibitor mixture at a final concentration of 1 mM and incubated for 20 minutes at 22 C prior to the enzyme assays. Small amounts of intrinsic amidolytic activity in the plasma

inhibitor solutions were subtracted from the remaining amidolytic activities of the enzyme-inhibitor mixture.

For study on thermal stability of the protease, inflamed skin extract at 24 hours was heated for 10 minute at various temperatures, and remaining amidolytic activities were measured.

Isoelectric Focusing

To estimate the isoelectric point of the protease, 1 ml of the preparation was dialyzed against 20 mM phosphate buffer (pH 7.4) at 4 C for 1.5 hours, and applied to an isoelectric focusing column (110 ml, Katoshoten, Osaka, Japan). The isoelectric focusing was performed with carrier ampholites (final concentration 1%, mixture of pH 3.5–10 (1 part) and pH 5–8 (4 parts) or pH 3.5–10 only) in a sucrose gradient. After focusing for 40 hours at 500 v, the designated volume of serial fractions was collected from the bottom of the column, the pH of each fraction was measured at 4 C, and the protease activity in each fraction was determined.

Sepharose 6B Column Chromatography

Two milliliter of skin extract were dialyzed against 20 mM phosphate buffer containing 100 mM NaCl (pH 6.5) at 4 C for 4 hours. The dialyzed sample was applied to a Sepharose 6B column (1.6 × 100 cm; bed volume, 200 ml) equilibrated with the same buffer. Protease fractions were pooled, concentrated 20-fold (to about 2 ml) by ultrafiltration with Diaflo membrane YM-10 (Amicon, Lexington, Ky), and used in the following experiments.

Sepharose CL-4B Column Chromatography and Polyacrylamide Disk Gel Electrophoresis of Radiolabeled Samples in the Presence of SDS

Six hundred microliters of the samples (Sepharose 6B pooled and concentrated fraction) were treated with 30 μl of ^{3}H-diisopropylfluorophosphate (1 mCi/200 μl) for 30 minutes at 37 C. Unreacted ^{3}H-diisopropylfluorophosphate was removed by exhaustive dialysis against 20 mM sodium phosphate buffer (pH 6.0) for 5 hours at 4 C and then against 20 mM sodium phosphate buffer containing 1% SDS and 8 M urea (pH 6.0) at room temperature for 12 hours.

For the column chromatography, 400 μl of the dialyzed sample was applied to a Sepharose CL-4B column (1.0 × 50 cm; bed volume, 40 ml) equilibrated with 20 mM sodium phosphate buffer containing 1% SDS and 8 M urea (pH 6.0). The radioactivity of each fraction (fraction volume, 400 μl) was counted in 10 ml of toluene scintillation fluid by the scintillation spectrometer (Packard Model 3385).

For SDS-polyacrylamide disk gel electrophoresis, 100 μl of each dialyzed sample was heated for 5 minutes at 100 C with or without β-mercaptoethanol, and 75 μl of each sample was applied to the SDS gels. The electrophoresis was performed by the method of Weber et al[15] with 8% gels. The gels were sliced into 1-mm pieces, and every two pieces of the slices were dissolved in 200 μl of 30% H_2O_2 by incubation for 12 hours at room temperature. The radioactivity of 100 μl of each sample was counted in 10 ml toluene scintillation fluid by the scintillation spectrometer (Packard Model 3385).

Marker proteins for calibration of the molecular weight were applied in the column and the disk gel under the same conditions.

Sepharose CL-4B Column Chromatography in the Presence of 6 M Guanidine Hydrochloride

Samples of the pooled and concentrated protease fraction from Sepharose 6B column chromatography were dialyzed against 20 mM sodium phosphate buffer containing 6 M guanidine hydrochloride (GuHCl) for 12 hours at 4 C. Two milliliters of the dialyzed sample was applied to a Sepharose CL-4B column (1.6 × 100 cm; bed volume, 200 ml) equilibrated with the same buffer. Each fraction (fraction volume, 2 ml) was dialyzed against 20 mM sodium phosphate buffer containing 200 mM NaCl (pH 5.0) for 12 hours at 4 C for removal of GuHCl; then the amidolytic activity in the fractions was measured by the same method as mentioned above. Marker pro-

Table 1 – Enzyme Specificity on Various Peptide-Methylcoumaryl Amide Substrates

Substrate (final concentration, 100 μM)	Amidolytic activity* (nM/min.)		Ratio§ (I/N)
	Inflamed†	Noninflamed‡	
Boc-Phe-Ser-Arg-MCA (trypsin)	908	33	27.5
Z-Phe-Arg-MCA (Plasma kallikrein)	240	175	1.37
Pro-Phe-Arg-MCA (Gland kallikrein)	188	140	1.34
Boc-Val-Leu-Lys-MCA (plasmin)	146	30	4.83
Bz-Arg-MCA (trypsin)	41	24	1.71

* The amidolytic activities were expressed as a velocity of aminomethylcoumarin release (nM/min).
† DHR skin at 24 hours.
‡ Unchallenged skin from an immunized animal as a control.
§ I/N, inflamed/noninflamed.

Table 2—Negligible Effect of Acetone Treatment to Protein Recovery and DHR Amidase Activity

	Acetone treatment	Protein concentration (A_{280})	Amidolytic activity* (nM/min)
Inflamed†	(+)	19.8	1463
	(−)	17.6	1208
Non-inflamed‡	(+)	14.8	62
	(−)	14.6	76

* The amidolytic activities were expressed as a velocity of aminomethylcoumarin release (nM/min).
† DHR skin at 36 hours.
‡ BGG-treated skin in normal animal at 36 hours as a control.

teins for calibration of the molecular weight were also eluted in the column under the same conditions.

Results

Trypsinlike Proteases in the Skin Extracts

Table 1 shows the amidolytic activity of the extracts from DHR sites at 24 hours (inflamed) and from unchallenged skin (noninflamed) on several common substrates for trypsinlike proteases. When the amidolytic activities were compared between inflamed skin and noninflamed skin (the I/N Ratio in Table 1), it was clear that Boc-Phe-Ser-Arg-MCA cleaving activity and Boc-Val-Leu-Lys-MCA cleaving activity were highly increased in the inflamed skin extract 28-fold and 5-fold, respectively. Since the former was more increased than the latter, and since the latter was assumed to be plasmin (or plasminlike protease) because of the amino acid sequence of the substrate, attention was focused on the Boc-Phe-Ser-Arg-MCA cleaving enzyme(s) in the following experiments. And unless otherwise specified, "amidolytic activity" henceforth means Boc-Phe-Ser-Arg-MCA hydrolytic activity.

To rule out unexpected effects of acetone treatment of the harvested skin on the recovery of the amidase, the amidolytic activities were compared between the extracts from the acetone-treated and from the non-acetone-treated skin. In this experiment DHR skin sites at 36 hours and BGG-treated skin sites at 36 hours in normal animals were used as inflamed and noninflamed, respectively. As shown in Table 2, the effect of acetone treatment of the harvested skin on the amidolytic activity was negligible.

Time Course Study of Amidolytic Activity

The chronologic relationship between the gross appearance of the inflammation and the amidolytic activity in the skin sites was investigated in DHR. As shown in Figure 1, the elevation of the amidolytic activity began at 9 hours, made a peak at around 36 hours, after the rapid increase, and gradually decreased but still remained elevated at 72 hours. Though the amidolytic activities in the extract from physiologic saline-treated skin site of the sensitized guinea pigs were elevated at 19, 36, 72 hours, the activities were very low in comparison with the DHR sites. Although slightly shifted to latter times when compared with the time course of the redness or the induration of the inflammatory site, the chronologic change of amidolytic activity in DHR seemed to be closely related to the inflammatory process.

Characterization of DHR Amidase

The skin extract from DHR sites at 24 hours was used as the source of the enzyme.

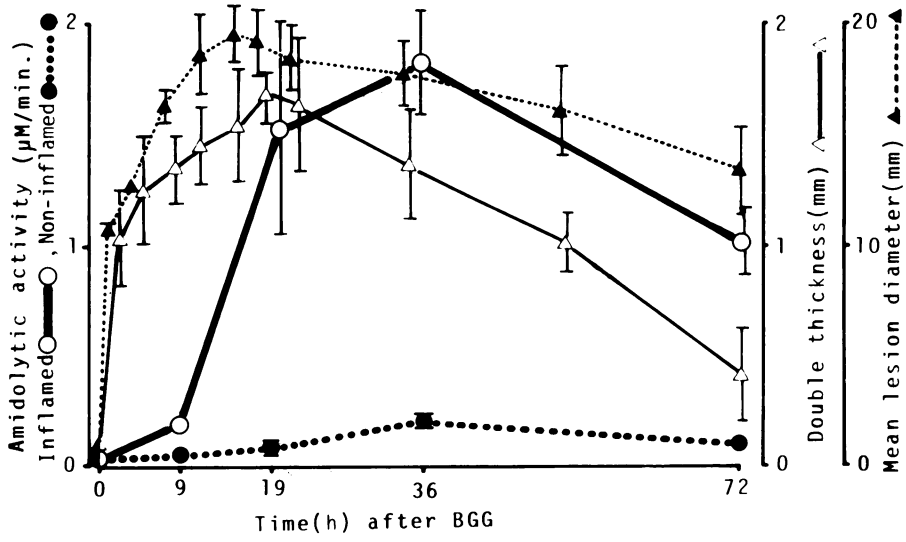

Figure 1—Time course of the amidolytic activity of the enzyme and delayed hypersensitivity inflammation. Amidolytic activities on Boc-Phe-Ser-Arg-MCA as substrate of inflamed (○——○) and physiologic saline-treated noninflamed control (●·····●) skin extracts. The double thickness (△——△) and mean lesion diameter (▲·····▲) of inflamed sites is also shown. BGG, bovine γ-globulin.

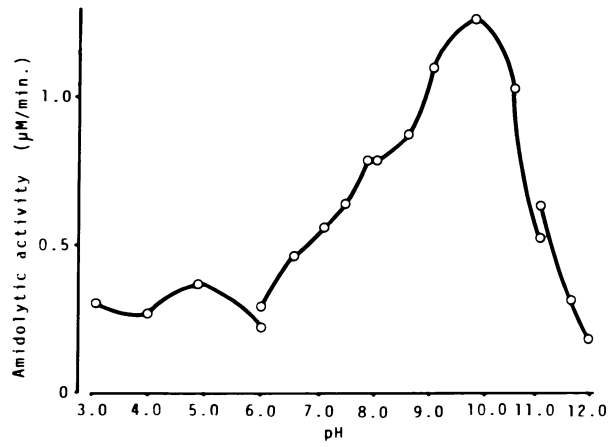

Figure 2 – pH curve of amidolytic activity of the enzyme. The buffers used were 200 mM CH_3COOH-CH_3COONa buffer (pH 3–6), 100 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 6–8), 200 mM Tris-HCl buffer (pH 8–11), 200 mM Na_2HPO_4-NaOH buffer (pH 11–12).

pH Dependency

The pH dependency profile of the amidolytic activity, shown in Figure 2, was the common shape for serine proteases, and optimum pH was around pH 10. Under the same conditions, an amidolytic activity of bovine pancreatic trypsin showed a shape similar to the pH profile with an optimum pH of 10.

Heat Stability

The amidolytic activity decreased to 23% when treated for 10 minutes at 56 C, and was almost abolished (only 1.2% remaining) when brought to over 80 C for 10 minutes. Thus, the amidase was heat-labile.

Isoelectric Point

The result of isoelectric focusing of the amidase is shown in Figure 3. The amidolytic activity was concentrated in a single peak at pH 4.2.

Molecular Weight

The molecular weight of the enzyme was estimated by Sepharose 6B column chromatography. As shown in Figure 4A, the amidolytic activity was eluted in a single peak between thyroglobulin and catalase as marker molecules, and the apparent molecular weight of the amidase was calculated as 600,000. The recovery of the activity in the fractions was approximately 90%. The extract from the control skin sites eluted in the same column showed negligible amidolytic activity in the corresponding fractions (data not shown). These results confirmed that the high-molecular-weight amidase was the very enzyme to which the amidolytic activity in DHR skin extract was attributed. This is unusually large in comparison with other trypsin-type proteases previously reported, with the exception of protease-α_2-macroglobulin complex. Even though the amidase had an apparent molecular weight smaller than that of guinea pig α_2-macroglobulin, the apparent pI was close to that of guinea pig α_2-macroglobulin (pI 4.6), and since many protease-α_2-macroglobulin complexes cleave small synthetic substrates (see discussion of trypsin-guinea pig α_2-macroglobulin complex below), it became critical to distinguish the high-molecular-weight amidase in DHR from a protease-α_2-macroglobulin complex. Since it is commonly known that protease-α_2-macroglobulin complex does not cleave high-molecular-weight substrates such as hemoglobin, the hemo-

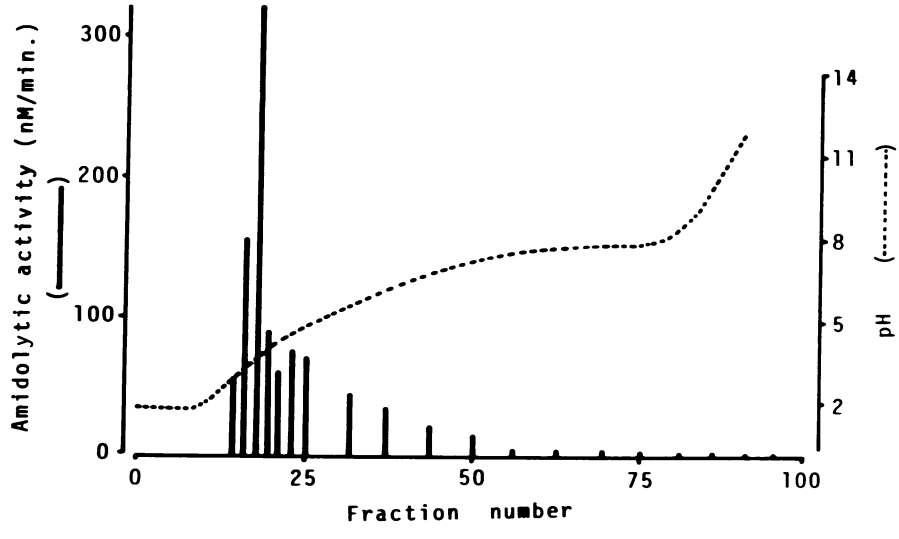

Figure 3 – Isoelectric focusing column of the 600K-protease. The thin broken line (• • • • • • •) denotes the pH gradient. The *solid bars* (▬▬) denote the potential amidolytic activities on Boc-Phe-Ser-Arg-MCA of 200 μl of each fraction (fraction volume, 1.5 ml).

globinolytic activities in the Sepharose 6B fractions were examined. Although the main hemoglobinolytic activity shown in Figure 4A was eluted in different fractions from the amidase activity with smaller molecular weight than BGG, a minor hemoglobinolytic activity was also observed coincident with the amidase activity peak. When the amidolytic fractions were pooled and rechromatographed on the same column, the hemoglobinolytic activity was coeluted with the amidolytic activity, making a single peak (Figure 4B).

These results suggest that there were two or more hemoglobinolytic neutral proteases in DHR skin extracts and that the high molecular weight amidase possibly hydrolyzed hemoglobin.

Differentiation of the High Molecular Weight Amidase From Protease-α_2-Macroglobulin Complex

Proteolytic Activity of the High Molecular Weight Amidase and Its Inhibitor Profile

To confirm that the hemoglobinolytic activity shown in Figure 4B was attributable to the amidase, sensitivity profiles to various protease inhibitors were investigated both for the amidolytic activity and for the hemoglobinolytic activity, and compared. As shown in Table 3, these sensitivity profiles were almost identical and seemed to be a common profile for trypsin-type proteases, with the exception of the resistance to tosyl-l-lysine-chloromethylketone and ovomucoid trypsin inhibitor. The results suggest that the high-molecular-weight amidase can also hydrolyze protein substrates like hemoglobin. This would be unusual for a protease-α_2-macroglobulin complex. It should also be noted on Table 3 that the high-molecular-weight protease was inhibited not only by the small inhibitors but also by relatively large protein inhibitors such as soybean trypsin inhibitor. This, too, would be unlikely for a protease-α_2-macroglobulin complex.

Effect of Anti-α_2-Macroglobulin Antibody

An immunoprecipitating effect of anti-guinea pig α_2-macroglobulin antibody was studied on trypsin-α_2-macroglobulin complex (as a positive control), and the high molecular weight amidase in the Sepharose 6B fraction. Briefly, the enzymes were incubated with antibody solution of varying dilutions or with the vehicle buffer at pH 7.4 for 20 minutes at 22 C and centrifuged to discard the immunoprecipitate, and the remaining amidolytic activities to Boc-Phe-Ser-Arg-MCA were measured. As shown in Table 4, though the amidolytic activity of trypsin-α_2-macroglobulin complex was almost completely precipitated or inhibited by antibody, the activity of DHR amidase was not affected.

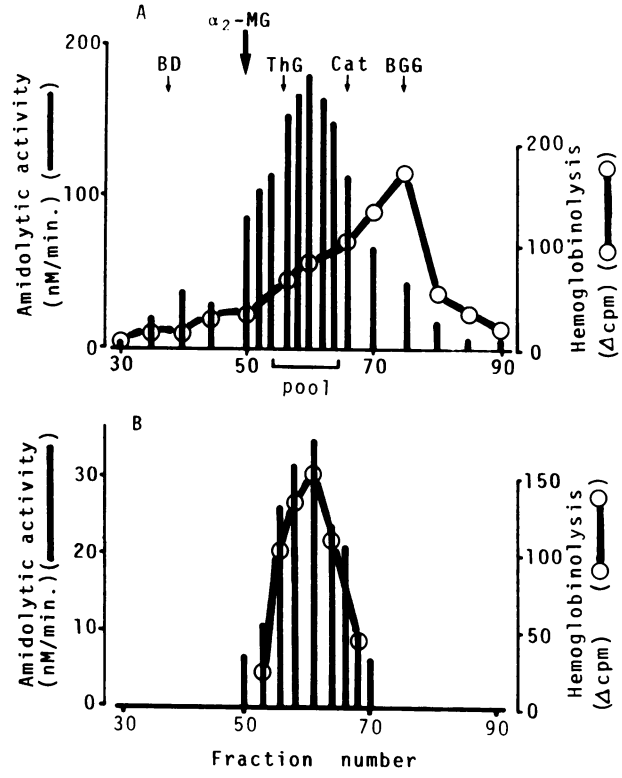

Figure 4A – The 600K-protease Sepharose 6B column chromatography. The *solid bars* (——) denote the amidolytic activities on Boc-Phe-Ser-Arg-MCA of 400 μl of each fraction (fraction volume, 2.0 ml). Hydrolytic activities on ^{3}H-acetyl hemoglobin of 50 μl of each fraction are also shown (○——○). α_2-MG, α_2-macroglobulin (mol wt 720,000); BD, blue dextran (mol wt 2,000,000); ThG, thyroglobulin (mol wt 680,000); Cat, catalase (mol wt 240,000); BGG, bovine γ-globulin (mol wt 150,000). The *horizontal bar* represents pooled fractions (55-65). **B** – Sepharose 6B column rechromatography of the pooled and concentrated fractions. The *solid bars* (——) denote the amidolytic activities at pH 10 on Boc-Phe-Ser-Arg-MCA of 400 μl of each fraction (fraction volume 2.0 ml). The hydrolytic activities at pH 8.5 on ^{3}H-acetyl hemoglobin of 50 μl of the same fractions are also shown (○——○).

From these results it was assumed that the high molecular weight protease in DHR site was not a complex of protease with α_2-macroglobulin.

The high-molecular-weight protease was arbitrarily named "600K-protease."

The 600K-Protease as an Oligomeric Molecule

To investigate whether the 600K-protease was a large monomeric protein or an oligomeric molecule, the protease in Sepharose 6B fraction was labeled in its active center with ^{3}H-diisopropylfluorophosphate and analyzed by SDS-polyacrylamide disk gel electrophoresis or by gel filtration column in the presence of SDS. As shown in Figure 5A, the ^{3}H-diisopropylfluorophosphate, which was assumed to be incorporated into the active center of the enzyme, appeared in a

Table 3—Susceptibility of the Enzyme to Various Protease Inhibitors

Protease inhibitor (Final concentration 1 mM)	Remaining activity**(%) Boc-Phe-Ser-Arg-MCA	Remaining activity**(%) ^{3}H-acetyl hemoglobin
None	100	100
Antipain	2	0
Leupeptin	3	0
Diisopropylfluorophosphate	16	0
Trasylol*	19	6
Phenylmethylsulfonylfluoride	22	15
Soybean trypsin inhibitor	31	22
Lima bean trypsin inhibitor	35	25
Benzamidine	56	43
p-Chloromercuribenzoate	75	73
N-Ethylmaleimide	88	76
Ethylenediaminetetraacetic acid-2Na	88	52
N-α-*p*-Tosyl-l-lysine-chloromethylketone-HCl	94	102
Ovomucoid trypsin inhibitor	103	158

The enzyme was incubated with indicated compounds at final concentration designated for 20 minutes at 37 C before the substrate (Boc-Phe-Ser-Arg-MCA or ^{3}H-acetyl hemoglobin) was added. Data are given as relative activity, taking the respective activities in the absence of inhibitors as 100%.

* Final concentration, 250 units.

position of 31,000 molecular weight in the SDS-polyacrylamide gel in the absence or in the presence of a reducing agent. Also as shown in Figure 5B, the radiolabeled ^{3}H-diisopropylphosphated protease was eluted with an apparent molecular weight of 33,000 from gel filtration column using Sepharose CL-4B in the presence of SDS.

These results suggested that the 600K-protease might be an oligomeric molecule with a core enzyme having an apparent molecular weight of 31,000–33,000. The core enzyme was arbitrarily named "31K-protease."

Table 4—Effect of Anti-Guinea Pig α_2-Macroglobulin-Rabbit Antibody

Sample +	Anti-α_2-MG-Ab* (fold dilution)	Amidolytic activity (nM/min)
600K-protease	(−)†	240
	× 1	220
	× 3	270
	× 10	270
	× 30	220
	× 100	220
Trypsin-α_2-MG‡	(−)†	208
	× 1	25
	× 3	16
	× 10	16
	× 30	11
	× 100	15

* Anti-guinea pig α_2-macroglobulin-rabbit IgG, A_{280} = 44.844, diluted with phosphate-buffered saline.

† Antibody was added.

‡ Trypsin-α_2-macroglobulin complex as a control.

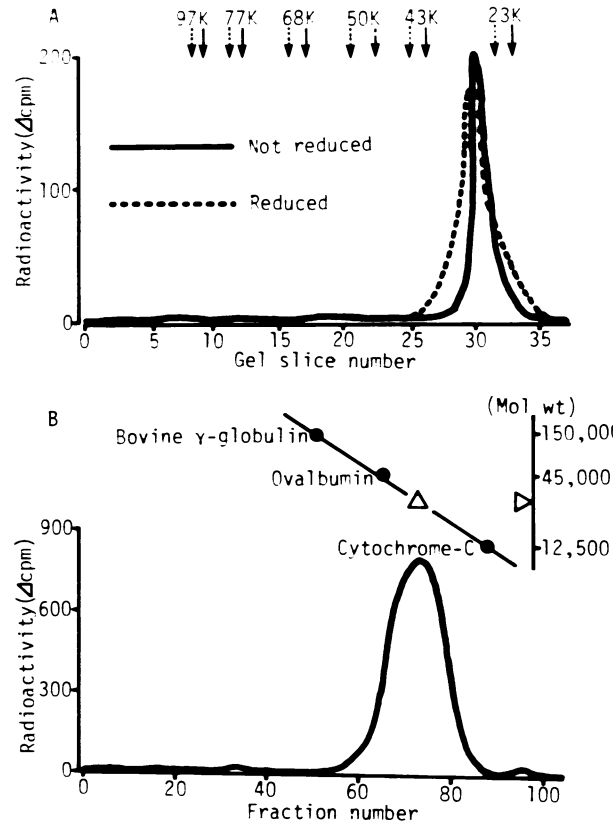

Figure 5A—SDS-polyacrylamide disk gel electrophoresis of ^{3}H-diisopropylfluorophosphate-labeled protease. The *thick line* (——) denotes nonreduced enzyme. The *broken line* (- - - -) denotes reduced enzyme. The locations of molecular weight standards are noted (K = 1,000 daltons). **B**—Sepharose CL-4B column chromatography of ^{3}H-diisopropylphosphated enzyme. Bovine γ-globulin (mol wt 150,000), ovalbumin (mol wt 45,000), and cytochrome-c (mol wt 12,500) are the marker proteins. The sign △ is the eluted enzyme peak and the ▷ is the protease position of molecular weight.

To rule out the possibility that the 600K-protease was large due to the complex formation with a protein(s) denatured during acetone treatment of the skin, the extract from non-acetone-treated skin of the DHR sites at 24 hours was gel-filtrated in the same Sepharose 6B column as used above. As shown in Figure 6, the elution profile of proteins was similar, and the bulk of amidolytic activity was eluted in the fractions corresponding to 600K-protease. In the column chromatography, another peak was observed in the void volume fraction with an apparent molecular weight more than 4,000,000, which was negligible in the case of acetone-treated skin. To date, detailed experiments have not been done for the amidolytic activity in the void volume fraction.

To investigate the binding strength of the oligomeric form of molecule, the 600K-protease radiolabeled with ^{3}H-diisopropylfluorophosphate was eluted in the gel filtration column in the presence of 2 M KCl or 6 M urea. The radioactivity, however, was not eluted

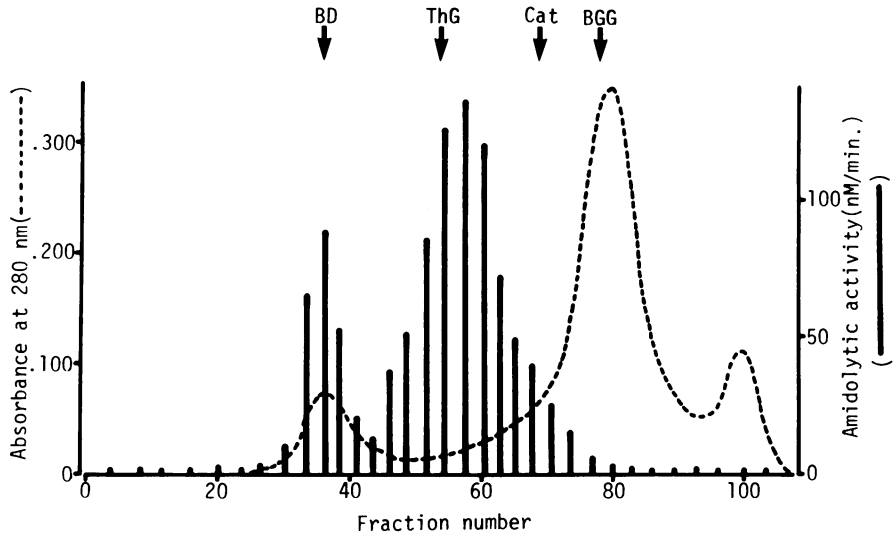

Figure 6—Sepharose 6B column chromatography of the DHR skin extract at 24 hours without acetone treatment. The *solid bars* (——) denote the amidolytic activity at pH 10 on Boc-Phe-Ser-Arg-MCA of 200 μl of each fraction (fraction volume, 2.0 ml). The *broken line* (- - -) denotes protein concentration determined from the absorbance at 280 nm (A_{280}). BD, blue dextran (mol wt 2,000,000); ThG, thyroglobulin (mol wt 680,000); Cat, catalase (mol wt 240,000); BGG, bovine γ-globulin (mol wt 150,000).

with the smaller molecular weight from the column, indicating that the complex was resistant to 2 M KCl or 6 M urea.

Separation of 31K-Protease in an Active Form

The 600K-protease fraction from Sepharose 6B column was chromatographed in the presence of 6 M GuHCl with the use of a Sepharose CL-4B gel filtration column. After dialysis of the fractions to remove GuHCl, the amidolytic activity of each fraction was measured. As shown in Figure 7, a single peak of the amidolytic activity was observed with an apparent molecular weight of 31,000, and the amidolytic activity corresponding to 600K-protease completely disappeared. The total recovery of amidolytic activity was approximately 10% of that of the 600K-protease before denaturation for the gel filtration chromatography. Fractions containing the renatured amidolytic activity were pooled, and the nature of the low-molecular-weight protease was analyzed.

As shown in Table 5, the enzymatic features of the low-molecular-weight protease, including substrate specificity, the pH dependency profile of the amidolytic activity, and sensitivity profile to exogenous protease inhibitors were almost identical to those of the 600K-protease, except for the high sensitivity of the former to *p*-chloromercuribenzoate. These results might confirm that the low-molecular-weight protease was the core 31K-protease in the 600K-protease molecule.

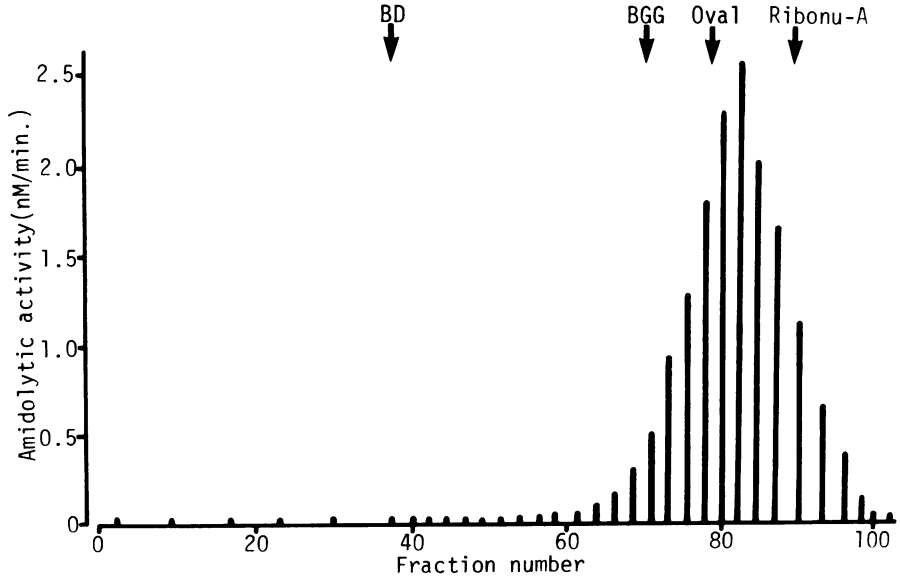

Figure 7—Sepharose CL-4B column chromatography of the 31K-protease in the presence of 6 M GuHCl. The *solid bars* (——) denote the amidolytic activity at pH 10 on Boc-Phe-Ser-Arg-MCA of 400 μl of each fraction (fraction volume, 2.0 ml). Each fraction was dialyzed to remove GuHCl and renature the protease to the active form before the amidolysis assay. BD, blue dextran (mol wt 2,000,000), BGG, bovine γ-globulin (mol wt 150,000); Oval, ovalbumin (mol wt 45,000); Ribonu-A, ribonuclease A (mol wt 13,700).

Table 5—Comparison of Enzymatic Properties Between 600K-Protease and 31K-Protease

	600K-protease	31K-protease
Substrate*		
Boc-Phe-Ser-Arg-MCA (Trypsin)	100%	100%
Z-Phe-Arg-MCA (Plasma kallikrein)	25	22
Boc-Val-Leu-Lys-MCA (Plasmin)	15	7
Optimum pH	pH 10	pH 10
Protease inhibitor†		
None	100%	100%
Leupeptin	2	7
Diisopropylfluorophosphate	16	14
Soybean trypsin inhibitor	31	23
Phenylmethylsulfonylfluoride	22	24
p-Chloromercuribenzoate	75	29
Ethylenediaminetetraacetic acid-2Na	88	83
N-Ethylmaleimide	88	85
Ovomucoid trypsin inhibitor	103	102

* The final concentration of each substrate was 100 μM. Data are given as relative activity, taking the respective activities as 100%, using the substrate, Boc-Phe-Ser-Arg-MCA.

† The final concentration of each protease inhibitor was 1 mM. Data are given as relative remaining activity, taking the respective activities in the absence of inhibitors as 100%. Both enzymes were incubated with the indicated compounds at the final concentration designated for 20 minutes at 37 C before the substrate, Boc-Phe-Ser-Arg-MCA, was added.

Isoelectric Point of 31K-protease

The result of isoelectric focusing of the 31K-protease is shown in Figure 8. The 31K-protease was a basic protein with an apparent pI 9.4, which was common for serine proteases reported of pancreatic or leukocytic origin.[16-18] It suggested that the 600K-protease with an apparent pI 4.2 was a complex of the 31K-protease and an acidic carrier molecule(s).

High Resistance of the 600K-Protease to Intrinsic Protease Inhibitors

To investigate the biologic significance of the high-molecular-weight form of the DHR amidase, we studied the sensitivity of the 600K-protease and the 31K-protease to intrinsic protease inhibitors in plasma, such as two kinds of trypsin inhibitor and α_2-macroglobulin. As shown in Figure 9, the 600K-protease was approximately 100 times more resistant to the plasma trypsin inhibitors than the 31K-protease. As shown in Table 6, the amidolytic activity of 31K-protease was protected by α_2-macroglobulin from the attack of soybean trypsin inhibitor, but the 600K-protease was not. This means that the 31K-protease might be bound to α_2-macroglobulin and rendered less susceptible to the inhibitor; and, on the other hand, the 600K-protease might be resistant to α_2-macroglobulin. Therefore, the 600K-protease was highly resistant to the intrinsic inhibitors in comparison with the 31K-protease, though the sensitiveness to extrinsic inhibitors of these two proteases was almost always identical (Table 5).

These results led us to assume that some of the pathophysiologic consequences of the complex formation might be to increase the resistance of the protease to intrinsic inhibitors and to maintain its activity longer *in vivo*.

Discussion

The present study, using recently developed amidolytic assays with fluorogenic peptide substrates, which have high sensitivity and specificity, revealed the presence of several trypsin-type proteases in the DHR

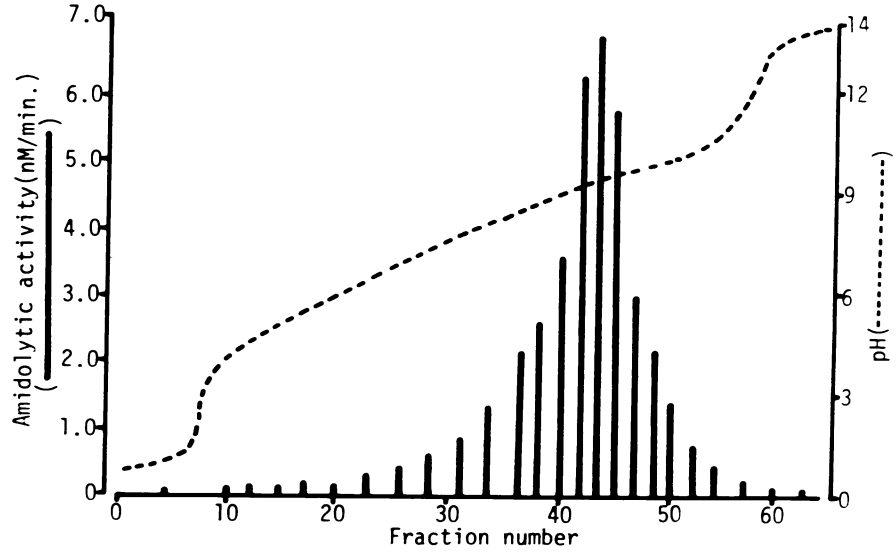

Figure 8—Isoelectric focusing of the 31K-protease. The *thin broken line* (·····) denotes the pH gradient. The *solid bars* (▬) denote the potential amidolytic activities at pH 10 on Boc-Phe-Ser-Arg-MCA of 400 μl of each fraction (fraction volume, 2.0 ml).

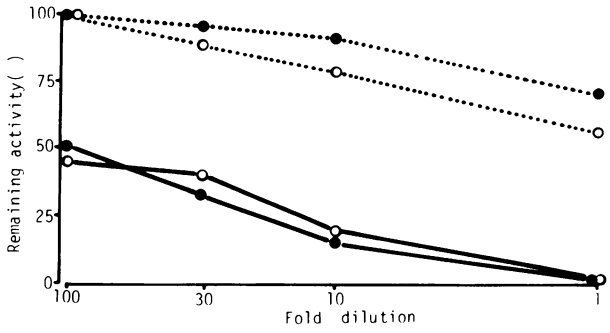

Figure 9—Comparison of susceptibilities between 600K-protease and 31K-protease to two kinds of plasma trypsin inhibitor (Ref. No. 9). Guinea pig plasma trypsin inhibitor-1 (●) (A_{280} = 2.45) and plasma trypsin inhibitor-2 (○) (A_{280} = 9.71) were logarithmically diluted with phosphate-buffered saline. The 600K- and 31K-protease solutions were incubated with the inhibitor solutions or with the vehicle for 20 minutes at 37 C before the amidolysis assay at pH 10. Data are given as relative remaining activity (after subtracting the small amounts of amidolytic activity contaminated in the inhibitor samples), taking the respective activities with the vehicle (3.41 nM AMC increase/min and 4.13 nM/min for the 600K-protease and 31K-protease, respectively) as 100%. The *broken lines* (- - -) denote 600K-protease and the *solid lines* (——) denote 31K-protease.

skin sites (Table 1). Of these proteases, the Boc-Phe-Ser-Arg-MCA cleaving enzyme had an extremely high molecular weight (600,000, 600K-protease) and an unusually acidic isoelectric point (pI 4.2), as compared with serine proteases previously reported,[16-18] although it had common patterns of trypsin-type protease for the pH dependency (Figure 2) and the inhibitor sensitivity profiles (Table 3). The possibility that the 600K-protease was a complex or an aggregation of protease(s) denatured during acetone treatment of the skin could be ruled out, since the 600K-protease was also observed in the extract of non-acetone-treated skin with similar amidolytic activity (Table 2; Figure 6). The 600K-protease could be also distinguishable from a protease-α_2-macroglobulin complex for the following reasons: 1) the 600K-protease was not precipitated by anti-guinea pig α_2-macroglobulin antibody, though trypsin-α_2-macroglobulin complex was (Table 4). 2) The 600K-protease was inhibited by relatively large protease inhibitors like soybean trypsin inhibitor (Table 3), although trypsin-α_2-macroglobulin complex was not. 3) The 600K-protease could hydrolyze substrate of high molecular weight, such as hemoglobin (Figure 4B; Table 3). 4) The apparent molecular weight of the 600K-protease was 120,000 daltons smaller than the guinea pig α_2-macroglobulin in the same gel filtration column (Figure 4A).

SDS-polyacrylamide gel electrophoretic analysis and gel filtration in the presence of SDS using ^{3}H-diisopropylphosphated form of the protease demonstrated that the 600K-protease might be an oligomeric molecule containing a core enzyme protein(s) with the apparent molecular weight of 31,000–33,000 (31K-protease) (Figure 5). Actually, a low-molecular-weight protease with an apparent molecular weight 31,000 was separated in an active form from the 600K-protease fraction by a gel filtration column in the presence of 6 M GuHCl (Figure 7). Recovery of the proteolytic activity from the denatured column was relatively low, however, the protease was identified with the core 31K-protease by the following: the apparent molecular weight was the same as the core 31K-protease; the substrate specificities, the pH dependency of the amidolytic activity, and the sensitivity profiles to extrinsic protease inhibitors, were identical to those of 600K-protease, except the 31K-protease was sensitive to *p*-chloromercuribenzoate (Table 5).

The sensitiveness to a thiol agent, *p*-chloromercuribenzoate, seems to be unusual for serine proteases. However, it was unlikely that the 31K-protease was a mixture of a serine protease and a thiol protease, since either diisopropylfluorophosphate or *p*-chloromercuribenzoate individually blocked more than 70% of the amidolytic activity. Since it is commonly believed that diisopropylfluorophosphate specifically binds to an active serine residue in the charge-relay system, but *p*-chloromercuribenzoate reacts not only to active cysteine but also to other cysteine residue if it is located outside of the molecule,[19] the 31K-prote-

Table 6—Comparative Study for Sensitivity to α_2-Macroglobulin Between 600K-Protease and 31K-Protease

Sample	Relative activity (%)
600K-protease + Buffer* + Buffer†	100
600K-protease + α_2-MG‡ + Buffer †	100.3
600K-protease + α_2-MG‡ + SBTI‡	21.9
600K-protease + Buffer* + SBTI‡	25.5
31K-protease + Buffer* + Buffer†	100
31K-protease + α_2-MG‡ + Buffer †	117.7
31K-protease + α_2-MG‡ + SBTI§	120.9
31K-protease + Buffer* + SBTI§	27.6

Proteases were incubated with α_2-macroglobulin (final concentration 250 μl/ml) or with the vehicle for 20 minutes at 37 C at pH 10, then incubated with soybean trypsin inhibitor (final concentration, 1 mM) or with the vehicle for 20 minutes at 37 C at pH 10 before the amidolysis assay at pH 10. Data are given as relative remaining activity (after subtracting the small amounts of amidolytic activity contaminated in the inhibitor samples), taking the respective activities with the vehicles (2.14 nM AMC increase/min and 2.72 nM/min for the 31K-protease and the 600K-protease, respectively) as 100%.

* 20 mM phosphate buffer, pH 6.5, containing 100 mM NaCl.

† 200 mM Tris-HCl buffer, pH 10.

‡ Guinea pig α_2-macroglobulin.

§ Soybean trypsin inhibitor.

ase should be classified as serine protease rather than thiol protease. Recently, presence of *p*-chloromercuribenzoate-sensitive diisopropylfluorophosphate-sensitive postproline cleaving enzyme,[20,21] and *p*-chloromercuribenzoate-sensitive calcium-dependent metal proteases, calpain or calcium-activated neutral proteases,[22-29] were reported in cellular proteases. We have also reported another *p*-chloromercuribenzoate-sensitive serine protease in lymph node lymphocytes in the immunized guinea pig.[30] It might be important to investigate the role of the thiol residue in the enzymatic functions as well as to determine whether this unique characteristic was specific for cellular proteases.

Of course, this characteristic does not prove that the 31K-protease is a cellular protease; and at the present time the origin of the protease is unknown. However, the size and the pI of the protease are consistent with the reports of cellular serine proteases, including those of leukocytic origin, but not plasma protease.[16-18] The assumption seems to be supported by the time course of the amidolytic activity in DHR sites, such that the appearance of increased amidolytic activity lagged slightly behind the intensity of redness or the induration (Figure 1), which suggests that the protease relates to cellular functions of the infiltrating leukocytes or other events at a middle or later stage in the inflammatory process rather than vascular responses in the early stage of the process. The research on trypsinlike proteases of leukocytes is not so advanced as chymotrypsinlike enzymes,[31] elastase or collagenase,[32,33] and the reports are few. Only Rothschild[34] reported an arginine esterase activity for rat mast cells. Judging from the time course, it is possible that the 31K-protease was of mononuclear cell (lymphocytes or macrophages) origin infiltrated in the lesion. The lymphocyte protease mentioned above seemed to be different from the present protease because of the resistance of the former to soybean trypsin inhibitor, trasylol, and benzamidine.[30] So far, we have not found a protease like the 31K-protease in guinea pig lymph node lymphocytes. To search for such proteases in macrophages or in culture supernatants of activated cells remains to be done. The next possibility for the origin might be the skin. Hatcher et al[35] reported a human skin protease with a molecular weight of 28,000, which included polymorphonuclear leukocyte infiltration by intradermal injection. It was inhibited by α_2-macroglobulin, α_1-protease inhibitor, C1-inhibitor, or other serine protease inhibitors. It would be of interest to know the relation between the human skin protease and the 31K-protease in the present study. Another hemoglobinolytic protease previously reported by us in DHR skin sites seemed different from the present enzyme because of the typical thiol-type protease of the former.[6] Song et al[36] reported a trypsinlike protease in irradiated guinea pig skin which liberated a bradykinin-kallidin-type vasoactive peptide from a precursor protein in the α-globulin fraction. We have reported a trypsinlike protease in guinea pig skin which was identical with Hageman factor in plasma and caused increased permeability when injected intradermally.[7,13,37-40] These two proteases should be discussed in relation to the early phase vascular reaction, if they have any role in the inflammatory process at all.

The apparent pI of the 31K-protease was 9.4 (Figure 8) and very different from that of the 600K-protease, 4.2 (Figure 3), suggesting that the latter might be a complex of the former with an acidic molecule(s). Recently, Fräki and his co-workers reported a protease-binding and -enhancing factor in normal human skin.[41-44] This factor, which was extracted with KCl containing buffer and had a molecular weight bigger than IgG, was able to complex with trypsin, chymotrypsin, or elastase, and enhanced trypsin hydrolytic activity at pH 8.0. The proteases in the complex preserved their hydrolytic activity toward protein substrates and were inhibited by soybean trypsin inhibitor. Furthermore, the binding factor did not react with anti-human α_2-macroglobulin antibody. The characteristics of the protease-factor complex in the report resemble the 600K-protease in the present paper, with the exception that the trypsin-binding factor complex was reported to be sensitive to treatment with high salt concentrations, such as 1 M NaCl. We did not succeed in separating the 31K-protease with either 2 M KCl or 6 M urea. Another trypsin- and chymotrypsin-binding factor was reported in rat plasma.[45] And Marossy reported the interaction between human granulocytic chymotrypsin- or elastase-like enzymes and glycosaminoglycans.[31] A conclusion on the identity or the relationship between these protease-binding molecules and the carrier molecule in the 600K-protease would be premature. However, since the 600K-protease was 100 times more resistant than the 31K-protease to intrinsic protease inhibitors, two kinds of trypsin inhibitor and α_2-macroglobulin, in guinea pig plasma (Figure 9, Table 6). There is a possibility that guinea pig binding factor, which might be the same as the carrier molecule in the present paper, was liberated in the inflammatory skin sites and protected the 31K-protease from attack by intrinsic protease inhibitors and made its activity last longer. And this might be one of the reasons why the 600K-protease was constantly recovered in the extract of the DHR lesion for such

a long period (up to 72 hours) in the active form (Figure 1).

The actual role of 600K-protease in the development or the regulation of DHR remains to be elucidated. It would be of great interest to know whether the 600K-protease or the 31K-protease generates or inactivates the macrophage-chemotactic factor from skin (MCFS-1). Further studies will address this question.

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