Partial Purification of Plasma and Tissue Kallikreins in Psoriatic Epidermis

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Human psoriatic scale extracts produced kinins from heated plasma (11.3 ± 5.5 ng kinin/mg protein) and from purified low molecular weight (LMW) bovine kininogen (4.4 ± 1.7 ng/mg). Sephacryl S-200 gel filtration of the extracts showed three peaks of kininogenase activity with M, values of 90,000 (K-I), 65,000 (K-II), and 35,000 (K-III). Upon DEAE-Sepharose chromatography of the Sephacryl peaks, K-I activity was found in the nonadsorbed fraction and formed kinins only from heated plasma. Peak K-II activity was resolved into two peaks, K-IIa (in the nonadsorbed fraction), which formed kinins only from heated plasma, and K-IIb (in the adsorbed fraction), which formed kinins from both heated plasma and LMW bovine kininogen. K-III kininogenase activity appeared at the same position as K-IIb and also formed kinins from both substrates. Kininogenases K-I and K-IIa had the same K_m value (0.3 mM) with Pro-Phe-Arg-p-nitroanilide(pNA), similar to that found with human plasma kallikrein. The K_m value of K-IIb with Val-Leu-Arg-pNA (0.8 mM) was like that found for human salivary kallikrein, whereas K-III had a low affinity for this substrate. Like plasma kallikrein, K-I and K-IIa were inhibited by soybean trypsin inhibitor, but only weakly by aprotinin. In addition the kininogenase activity of both K-I and K-IIa was neutralized by adding antihuman prekallikrein immunoglobulin G (IgG). In contrast, K-IIb and K-III were strongly inhibited by aprotinin but not by soybean trypsin inhibitor, consistent with their being tissue kallikreins. It was confirmed that K-IIb and K-III shares antigenic determinant of urinary kallikrein. J Invest Dermatol 90:505–510, 1988

Psoriasis is a chronic skin disease characterized by inflammation of the involved skin, as shown by edema of papillae, dilatation of papillary capillaries, and infiltration of leukocytes into the epidermis, as well as accelerated turnover of the epidermal cells [1]. In psoriatic skin, elevated levels of proteinases have been found. Haustein [2] reported strong plasminogen activator (PA) activity in the parakeratotic area of the epidermis using fibrin autography technique. Fraki and Hopso-Hasu [3] extracted PA, a trypsinlike esterase, a chymotrypsinlike esterase, and a cathepsin B-like cysteine proteinase. Furthermore, Hibino [4] purified a keratin-hydrolyzing proteinase with M, 30,000 from psoriatic scale extract. However, normal cornified cell extracts did not contain proteinase activity [4,5], suggesting that proteolytic activation in the epidermis plays a role in the pathogenesis or modulation of the psoriatic condition.

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Abbreviations:
- DFP: diisopropyl fluorophosphate
- HMW: high molecular weight
- LMW: low molecular weight
- PA: plasminogen activator
- PBS: phosphate buffered saline
- pNA: p-nitroanilide
- SBTI: soybean trypsin inhibitor
- TCA: trichloroacetic acid

Materials Scales (20 g) were collected from 15 patients with psoriasis vulgaris, between the ages of 18 and 69 yr, of both sexes. The patients had been diagnosed by clinical and histological criteria in Iwate Medical University Hospital and in the Medical Institute of Bioregulation Hospital, Kyushu University, Beppu.

Extraction and Separation of Kininogenases In each experiment, 3.5–5.0 g of psoriatic scales were homogenized with glass homogenizers and extracted with 0.1 M Tris–HCl + 0.14 M NaCl (pH 8.0) for 2 h at 4°C. The supernatant solution from centrifugation at 33,000 g for 1 h was concentrated with an Amicon YM 10 membrane and applied to a Sephacryl S-200 (Pharmacia, Piscataway, New Jersey) column equilibrated with 0.02 M sodium phosphate + 0.14 M NaCl, pH 7.5 (PBS). The kininogenases were further separated by DEAE-Sepharose (Pharmacia) chromatography equilibrated with 0.02 M sodium phosphate buffer, pH 7.5. M, values of the separated enzymes were estimated by Superose 12 (Pharmacia) FPLC in the presence of PBS.

Assay for Kininogenases Kininogenase activity was measured by the ability to release kinins. Human plasma served as the substrate for both plasma and tissue kallikreins after incubation at 60°C for 30 min in the presence of 4 mM 1,10-phenanthroline. Purified bo-
vine LMW kininogen (Seikagaku Kogyou, Tokyo, Japan) was used as another kininogenase substrate for tissue kallikrein. Samples (100 µl) and 0.2 M Tris–HCl (pH 8.5) (50 µl) were incubated with 50 µl of heated plasma or LMW kininogen (0.5 mg/ml) for 60 min at 37°C. After addition of 50 µl of 0.15% trichloroacetic acid (TCA), the mixture was cooled in an ice box for 30 min. Kinins in the supernatant solutions were assayed by a competitive enzyme immunoassay method [7]. A test sample containing unlabeled kinins was mixed with galactosidase-labeled bradykinin and primary antibody immunoblot. The immune complexes were trapped by secondary antibody immobilized on bacterial cell walls. The galactosidase activity in the precipitate was measured using p-nitrophenyl-galactoside (Markit A Bradykinin, Dainippon Pharmaceutical, Osaka, Japan). The linearity of measurement between the detection limit (1 ng of kinins/assay tube) and the maximum (20 ng/assay tube) was confirmed. In this condition, excess amounts of pancreatic kallikrein (0.01 or 0.1 mg/ml) (purified by Showa Denko, Tokyo) yielded more than 160 ng kinins from both human plasma and LMW bovine kininogen, while excess trypsin (0.083 or 0.83 mg/ml) (Sigma, type III) produced more than 40 ng kinins from both substrates. In addition, kininogen content in psoriatic scale extracts was measured as kinins. Psoriatic scale extract (100 µl) was incubated with purified porcine pancreatic kallikrein (0.02 U/ml) (a gift from Showa Denko) for 60 min at 37°C. After the addition of TCA, the liberated kinins were assayed as described above. Free kinins in the psoriatic scales were also measured after precipitation with TCA.

**Figure 1.** Sephacryl S-200 gel chromatography of the crude extract of psoriatic scales. Column: 3.2 X 85 cm; fraction volume: 5.5 ml. (A) Kininogenase activity with heated plasma (filled triangles) and with bovine LMW kininogen (open triangles). Three fractions (K-I, K-II, and K-III), shown by bars, were collected. (B) Hydrolysis of S-2288 (open circles), S-2266 (filled circles), and S-2302 (filled squares) were shown.

**Synthetic Substrate Assay** Enzyme samples (50 µl) in 0.9 ml of buffer were incubated with 50 µl of 4 mM Ile-Pro-Arg-pNA (S-2288) (Kabi, Stockholm, Sweden), which has a relatively broad specificity for trypsin-like serine proteinases, Val-Leu-Arg-pNA (S-2266) (Kabi), a substrate developed for tissue kallikrein, or Phe-Arg-pNA (S-2302) (Kabi) for plasma kallikrein, at 37°C, respectively. The reaction was stopped by adding 0.1 ml of acetic acid, and the absorbance at 405 nm was measured. The buffers used were 0.05 M Tris–HCl (pH 8.5) for S-2288 and S-2266 assays, and 0.05 M Tris-HCl (pH 7.5) for S-2302. Activity was expressed as nanomoles per minute per milliliter of p-nitroaniline liberated, assuming ε₄₀₅ = 10,500.

**Effect of Difosphophfluorophosphate (DFP), Soybean Trypsin Inhibitor (STI) and Aprotinin** The kininogenase concentration of each fraction from the DEAE Sepharose column was adjusted to liberate about 10 ng of kinins in the enzyme immunoassay. Kininogenase (50 µl) was incubated with 50 µl of PBS containing various concentrations of DFP, SBTI, or aprotinin for 30 min at 25°C. Inhibition was expressed as percent decrease in liberation of kinins.

**Effect of Antibodies to Kininogenases** Immunoglobulin of goat antiserum to human plasma prekallikrein (Nordic Immunological Laboratory, Amsterdam) was precipitated in 50% saturation of ammonium sulfate, and further purified by DEAE Sepharose chromatography. Rabbit antiserum to human urinary kallikrein (Japan Chemical Research, Tokyo) was purified through protein
A-Sepharose. The IgG fraction was passed through aprotinin-Sepharose column before use. The IgG solution in PBS (50 μl) of antiplasma prekallikrein (3 mg/ml) or antiurinary kallikrein (0.5 mg/ml) was mixed with 50 μl of enzyme sample. After 30 min at 25°C, 50 μl of 0.2 M Tris-HCl containing 4 mM 1,10-phenanthroline (pH 8.5), and 50 μl of heated plasma were added and incubated 60 min at 37°C. Kinins in the supernatant after TCA precipitation were assayed. Human plasma kallikrein (Sigma) and human urinary kallikrein (Japan Chemical Research) were used for control.

Protein Concentration was determined by the method of Lowry et al [8] using bovine serum albumin as standard.

RESULTS

Detection of Kininogenase, Kininogen, and Kinins Tris-buffered saline extracts of psoriatic scales hydrolyzed S-2288 (4.9 ± 1.4 nmol/min/mg protein; mean ± SD; N = 4), S-2266 (2.1 ± 1.0), and S-2302 (3.1 ± 2.0). Psoriatic scale extracts gave kininogenase activity with heated human plasma (11.3 ± 5.5 ng/mg protein). However, the extracts released kinins less effectively from LMW kininogen (4.4 ± 1.7). Neither endogenous kininogen nor kinin was detectable in the extracts.

Separation of Kininogenases Figure 1 shows the Sephacryl S-200 chromatographic elution diagram of concentrated crude extract. Kininogenase activity with heated plasma was found in three fractions: M, 70,000–100,000 (K-I), M, 50,000–70,000 (K-II), and M, 25,000–40,000 (K-III). K-I forms kinins only from heated plasma but not from LMW kininogen. A peak of S-2302 hydrolytic activity coincided with the kininogenase activity. K-II showed lower kininogenase activity with LMW kininogen, while K-III had the highest kininogenase activity, and the same specific activity with both heated plasma and LMW kininogen. Peptidolytic activity for S-2302 was highest in K-I, though activity for S-2288 and S-2266 was highest in K-III.

In the subsequent DEAE-Sepharose chromatography of K-I (Fig 2), the kininogenase activity with heated plasma was detected in nonadsorbed fraction, but not with LMW kininogen. The kininogenase activity of K-II was separated into two peaks (Fig 3). K-IIa in the nonadsorbed fraction formed kinins from heated plasma but not from LMW kininogen. On the other hand, K-IIb eluted with 0.4 M NaCl showed kininogenase activity with both heated plasma and LMW kininogen, and was relatively less active with S-2302. As shown in Fig 4, the kininogenase activity of K-III appeared at the same position as K-IIb and released kinins from both substrates. S-2288 and S-2266 hydrolytic activities in K-III were found low level, while a nonspecific peptide hydrolytic activity for S-2288 and S-2266 was eluted separately. Specific kininogenase activity increased eight- to eightyfold (Table I). Superose 12 FPLC showed M, values of 90,000 for K-I, 65,000 for K-IIa and K-IIb, and 35,000 for K-III, respectively.

Km Values for Psoriatic Scale Kininogenases The Km values toward synthetic peptide substrates were determined using Lineweaver–Burk plots (Table II). K-I and K-IIa demonstrated the same value with S-2302 (0.3 mM). The Km values of K-IIb and K-III with S-2266 were considerably different, although these enzymes had similar values with S-2288 (0.6 and 0.8 mM, respectively).

Effect of DFP, SBTI, and Aprotinin on Kininogenase Activity K-I, K-IIa, K-IIb, and K-III were all serine proteases that were inactivated by 1 mM DFP (100%, 100%, 96%, and 72%, respectively). Figure 5 showed the effect of SBTI and aprotinin on kininogenases. Whereas K-I and K-IIa were inhibited more than 40% by 1 μg/ml of SBTI, demonstrating similar inhibition profiles, 100 μg/ml of SBTI did not inhibit K-IIb and K-III. On the other hand, K-I and K-IIa were partially inactivated by 33 U/ml of aprotinin, while K-IIb and K-III were inhibited more than 80% by this concentration of aprotinin.

Neutralization of Kininogenase Activity by Antibodies Antihuman plasma prekallikrein IgG inhibited control plasma kallikrein but did not inactivate human urinary kallikrein (Table III). In this experimental condition, the IgG inhibited kininogenase activity of K-I and K-IIa, respectively, although it inactivated neither K-IIb nor K-III. On the other hand, antiurinary kallikrein IgG inhibited control urinary kallikrein but showed no effect on plasma.

[Image of Figure 2: DEAE-Sepharose chromatography of K-I. The column (1.6 × 25 cm) was equilibrated with 0.02 M sodium phosphate buffer, pH 7.5, and eluted with increasing concentration of NaCl (dashed line) in the same buffer. (A) Kininogenase activity with heated plasma (filled triangles) and with LMW kininogen (open triangles). (B) Hydrolysis of S-2288 (open circles) and S-2302 (filled squares) were also measured (fraction volume: 2.7 ml).]
**Figure 3.** DEAE-Sepharose chromatography of K-II. (A) Kininogenase activity from heated plasma (filled triangles) and LMW kininogen (open triangles). Two peaks (K-IIa and K-IIb) were separated as indicated by bars. (B) Hydrolysis of S-2288 (open circles), S-2266 (filled circles), and S-2302 (filled squares) were also shown (fraction volume: 2.7 ml).

**Figure 4.** DEAE-Sepharose chromatography of K-III. (A) Kininogenase activity with heated plasma (filled triangles) and with LMW kininogen (open triangles). (B) Hydrolysis of S-2288 (open circles) and S-2266 (filled circles) (fraction volume: 2.7 ml).
Table I. Summary of Purification of Kallikreins in Psoriasis

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<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Protein Concentration (mg/ml)</th>
<th>Kininogenase Activity (ng/0.1 ml)</th>
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<th>Specific Activity (ng/mg)</th>
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Table II. $K_w$ Values of Kininogenases

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<th>S-2266</th>
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<tr>
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<td>—</td>
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<tr>
<td>K-IIa</td>
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<tr>
<td>K-IIb</td>
<td>—</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>K-III</td>
<td>—</td>
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</table>

*Expressed as $10^{-8}$ M.

Kallikrein. The IgG inhibited neither K-I nor K-IIa, while it completely inactivated K-IIb and K-III. It was confirmed that kininogenase activity in the psoriatic scale extracts is due to the action of both plasma kallikrein (K-I and K-IIa) and tissue kallikrein (K-IIb and K-III).

DISCUSSION

Psoriatic scale extracts contained four species of serine proteinases able to release kinins. Among these, kinin-liberating enzymes, K-1 and K-IIa released kinins only from heated plasma but not from LMW kininogen, and were inhibited by SBTI and aprotinin, indicating that they are plasma kallikreins. The $K_w$ values of K-I and K-IIa with S-2302 were similar to that of plasma kallikrein (0.2 mM) [11]. In addition, both K-I and K-IIa were inactivated by antibody to plasma prekallikrein. K-I (M, 90,000) and plasma kallikrein (M, 88,000 and 86,000) [12] have similar M, values, respectively, while the smaller M, value of 65,000 of K-IIa may mean that it is a degraded plasma kallikrein.

Like tissue kallikreins [9,10], K-IIb and K-III liberated kinins from bovine LMW kininogen as well as from heated plasma, and K-IIb and K-III were strongly inhibited by aprotinin, but not by SBTI. Tissue kallikreins have been purified from salivary glands, pancreas, and urine [13,15]. The $K_w$ value of K-IIb with S-2266 (0.8 mM) was very similar to that of salivary kallikrein (0.6 mM), but it was different from that of pancreatic kallikrein (0.021 mM), or urinary kallikrein (0.024 mM). Plasmmin, which is known to liberate kinins from both HMW and LMW kininogen, was not found in this fraction. Kininogenase fractions including K-IIb did not show any effect on fibrin, and the hydrolytic activity with S-2251 (a substrate for plasmin in the same S-series peptide chromogenic substrates from Kabi), was undetectable (unpublished data). By use of antibody to urinary kallikrein, however, it was shown that K-IIb is related to urinary kallikrein. K-III differed from K-IIb in molecular weight and $K_w$ value with S-2266. Keratin hydrolyase, recently purified from psoriatic epidermis, showed about the same molecular weight as K-III and substrate specificity for peptide substrates [4]. However, this enzyme is not contained in K-III because keratin hydrolyase is inhibited by SBTI, although K-III was not. It was confirmed that K-III has the same antigenic determinant to urinary kallikrein.

Kallikreinlike activity has been shown by Fox and Hilton [16] and Frewin et al [17] in human eccrine sweat. Recently, Hibino et al [18] demonstrated that fresh sweat contains two kinds of tissue kallikreins with M, 45,000 and 35,000. The enzyme with M, 35,000 showed higher hydrolytic activity with S-2288 than with S-2266, indicating similarity with K-III of the present study. Toki and Yamura purified a kallikreinlike enzyme from human skin extract [19]. This enzyme, with M, 100,400, was inhibited by tosyl-lysine chloromethyl ketone (TLCK), $\alpha$-antitrypsin, and SBTI. This enzyme was immunologically distinct from human plasma, or urinary or pancreatic kallikreins. Furthermore, the fact that this enzyme was extractable only by high salt showed a marked differ-

Figure 5. Effect of SBTI and aprotinin on kininogenases. K-I (filled circles), K-IIa (open circles), K-IIb (filled triangles), and K-III (open triangles) were incubated with various concentrations of SBTI (A) or aprotinin (B). The free kininogenase activity was measured by enzyme immunoassay and percent inhibition was plotted.
ence from plasma kallikrein or K-I. Miller et al reported that leukocytes contain a neutral proteinase capable of liberating kinins [20]. However, this activity is plasminogen-dependent, showing that the enzyme is not a kallikrein. A neutral proteinase that can liberate kinins can be isolated from leukocytes [21]. This proteinase had the properties of elastase.

The kallikrein–kinin system has a close relationship with the complement system. Plasma kallikrein activates C1s directly [22] and Vogt et al [23] reported that this enzyme enhanced the release of C5a. Furthermore, plasma kallikrein activated by Hageman factor generates plasmin [24], which is known to trigger the classical pathway of complement system through the activation of C1. Plasmin also has a direct action on C3 or C5 to release chemotactic factors C3a and C5a [25]. In psoriasis, activation of the complement system has been shown by the infiltration of neutrophils into the epidermis. Extracts of complement degradation products from psoriatic scales show leukotactic activity [26]. Lazarus et al [27] found a serine proteinase in extracts of psoriatic plaque that causes leukotaxis in presence of complement. Activation of the kallikrein–kinin system may account for the inflammatory reactions observed in psoriatic skin. The present study revealed that multiple forms of kallikrein are functionally active in the psoriatic epidermis and may play important roles in the pathogenesis of psoriasis.

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REFERENCES

Table III. Effect of Antibodies on Kinogenase Activity

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<th>Antibody</th>
<th>Plasma Kallikrein</th>
<th>Urinary Kallikrein</th>
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<th>K-Ib</th>
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<tbody>
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* Expressed as percentage of inhibition.