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Mast cell chymase degrades fibrinogen and fibrin

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Conflicts of interest: None to declare.

What's already known about this topic?

- The accumulation of immunoreactants and fibrinoid necrosis of postcapillary vessel walls are common pathological features of cutaneous immune complex vasculitis.
- Mast cell chymase degrades complement C3 and C3a
- Heparin can influence the catalytic properties of chymase.

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What does this study add?

- Immunofluorescence staining positivity of fibrin in vasculitis cryosections decreased after pre-treatment with rh-chymase for 24 h; heparin enhanced this effect
- Rh-chymase degraded the alpha-, beta- and gamma-chains of fibrinogen, while heparin enhanced the degradation of beta-chain.
- Rh-chymase pre-treatment of fibrinogen prolonged thrombin-induced clotting time and fibrinogen degradation products induced by rh-chymase prolonged the clotting time of human plasma.
- Rh-chymase degraded fibrin gel prepared from fibrinogen or human plasma.

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Summary

Background: The accumulation of immunoreactants and fibrinoid necrosis of postcapillary vessel walls are common pathological features of cutaneous immune complex vasculitis. In more advanced lesions, these immunoreactants are subject to proteolysis. Mast cell chymase is a powerful enzyme that can degrade several substrates including the extracellular matrix. Heparin can influence the catalytic properties of chymase.

Objectives: We aimed to study the effects of recombinant human (rh) chymase on fibrinogen, coagulation and fibrinolysis, and to relate these effects to the vasculitis pathogenesis.

Methods: The co-localization of chymase and fibrin in vasculitis specimens was analysed by immunohistochemical double-staining. Fibrinogen and fibrin were treated with rh-chymase and the effects were studied *in vitro* by SDS-PAGE electrophoresis and a variety of clotting and fibrin gel experiments. The effects of rh-chymase on vasculitis cryosections were analysed by direct immunofluorescence (IF).

Results: Chymase-positive mast cells associated with fibrin-positive vessels in vasculitis cryosections. Rh-chymase degraded the alpha-, beta- and gamma-chains of fibrinogen, while heparin enhanced the degradation of the beta-chain. Rh-chymase pre-treatment of fibrinogen prolonged thrombin-induced clotting time. Fibrinogen degradation products induced by rh-chymase increased the clotting time of human plasma. Rh-chymase degraded fibrin gel prepared from fibrinogen or human

plasma. IF staining positivity of fibrin in vasculitis cryosections decreased after pre-treatment with rh-chymase for 24 h and heparin enhanced this effect.

Conclusions: Mast cell chymase may constitute a previously unrecognized endogenous anticoagulant and fibrinolytic enzyme which may be involved in the clearance of fibrin from vessel walls in aged vasculitis lesions.

Introduction

The initiating event in the pathogenesis of cutaneous immune complex vasculitis of small vessels is the precipitation of circulating immune complexes in the postcapillary vessel walls; this event results in the activation of the complement system, infiltration of neutrophils, leukocytoclasia and fibrinoid necrosis of the vessel wall. Consequently, vascular immunoglobulins, complement products and fibrin can be detected with direct immunofluorescence (IF) staining presently used in vasculitis diagnostics.[1,2]

In normal skin, mast cells are located in the upper dermis and around vessels, skin appendages and nerves. The vast majority of cutaneous mast cells belong to the MC_{TC} subtype containing tryptase, chymase, carboxypeptidase and cathepsin G in the secretory granules.[3] Tryptase and chymase are complexed to heparin proteoglycan.[4] Enzymatically active β-tryptase is a ring-like tetrameric enzyme that needs heparin for stability. Chymase is a monomeric enzyme that also binds to heparin, although heparin is not needed for stability. However, heparin can influence the catalytic properties of chymase.[5–7] Under physiological conditions chymase is bound to heparin. In contrast to tryptase, large chymase-heparin proteoglycan complexes appear to diffuse slowly in the extracellular matrix .[5,8] We have recently found that the vascular IF positivity of fibrin and complement C3c correlates positively with mast cells containing chymase activity in the initial petechial (IP) lesion of cutaneous immune complex vasculitis. In the more advanced lesion (i.e. the palpable purpura (PP) lesion) the IF positivity of fibrin correlated positively with mast cells containing tryptase and chymase immunoreactivities, though chymase is partially inactivated in the PP lesion. These results point towards a possible role for chymase in vasculitis pathogenesis.[9]

Fibrinogen is a 340-kDa glycoprotein consisting of pairs of alpha-, beta- and gamma-chains. Fibrin(ogen) has several different binding sites for plasma proteins and cellular receptors which explains its many functions, including; binding of thrombin in fibrin gel formation and blood clotting, plasmin inhibitor α_2-PI in fibrinolysis, or leukocyte integrin $\alpha_M\beta_2$ (CD11b, Mac-1) in inflammation.[10,11] Human tryptase has been shown to degrade alpha- and beta-chains of fibrinogen.[12–14] However, tryptase is unable to substantially solubilize cross-linked fibrin *in vitro.*[14] Chymase can degrade the extracellular matrix, activate procollagenase, cause epidermis-dermis separation[15–17] and degrade complement C3 and C3a.[18] However, information on the possible effects of human mast cell chymase on fibrinogen, coagulation and fibrinolysis is sparse.

In the present study we analysed immunohistochemically the co-localization of chymase and fibrin in vasculitis specimens, and examined whether the treatment of vasculitis cryosections with recombinant human (rh) chymase can affect the IF-positivity of fibrin, similar to complement C3c.[18] *In vitro*, fibrinogen and fibrin were treated with rh-chymase and the effects were studied using SDS-PAGE electrophoresis and a variety of clotting and fibrin gel experiments. The results suggest that chymase may constitute a previously unrecognized endogenous anticoagulant and fibrinolytic enzyme.

Materials and methods

Chemicals

Rh-chymase, fibrinogen, thrombin, chymostatin, tosyl-lycine chloromethyl ketone (TLCK), Tris-HCl, and heparin glycosaminoglycan sodium salt from porcine intestinal mucosa (reagent suitable for cell culture, Mr approximately 9-21 kDa, with the greatest amount centering between 17-19 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents for immunohistochemistry were purchased from Vector Laboratories (Burlingame, CA, USA) and the mouse anti-human chymase monoclonal antibody (mAb) was from SeroTec (Oxford, UK). The rabbit polyclonal FITC-labelled anti-human fibrinogen antibody (code F0111) used for IF staining purposes, and the polyclonal anti-human fibrinogen antibody (code A0080) for the immunohistochemical double-staining, were both purchased from Dako Denmark A/S (Glostrup, Denmark).

Sequential double-staining for chymase and fibrinogen

The association between mast cell chymase and fibrinogen was determined with a double-staining method. Biopsies were taken from healthy, IP and PP skin of 10 patients with immune complex-mediated and histopathologically confirmed leukocytoclastic vasculitis of small vessels.[9,18] Cryosections (5 µm) of the biopsies were first stained immunohistochemically for mast cell chymase using 1 µg/ml anti-chymase mAb and the Vectastain ABC-AP Mouse IgG kit to visualize mast cells as red. The same sections were stained immunohistochemically for fibrinogen using 0.79 µg/ml polyclonal anti-human fibrinogen and Vectastain Elite ABC Rabbit kit with 0.05% 3,3´-diaminobenzidine, 0.04% nickel chloride, and 0.03% hydrogen peroxide. Mouse IgG_1 Isotype Control (1 µg/ml, R&D Systems) was used as negative control for chymase and Rabbit IgG Isotype Control (0.79 µg/ml, Novus) for fibrinogen. Chymase$^+$ mast cells were counted within an area of 1.0 mm (width) x 0.6 mm (depth) in the upper dermis just beneath the epidermis from two sections of each sample. The percentage of chymase$^+$ mast cells in close association with fibrinogen$^+$ vessels was determined.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of rh-chymase-treated fibrinogen

The degradative effect of rh-chymase on fibrinogen was studied with SDS-PAGE electrophoresis under reducing conditions. Fibrinogen (1.13 mg/ml in PBS) was incubated with 0, 1 and 10 µg/ml rh-chymase at 37 °C in the absence or presence of 20 µg/ml heparin. The reaction was stopped after 2 h incubation with 20 % trichloroacetic acid (TCA) followed by SDS-PAGE electrophoresis under reducing conditions using 12.5% SDS-gels, as described previously.[18]

Immunofluorescence staining of fibrinogen in vasculitis cryosections after treatment with rh-chymase and heparin

Ten skin samples of small vessel vasculitis from the hospital archives of diagnostic biopsies were chosen for experimentation. Cryosections (5 µm) were cut onto glass, washed in PBS, and then incubated with 10 µg/ml rh-chymase, 10 µg/ml rh-chymase and 20 µg/ml heparin, or diluent control (PBS) in a humidified chamber at room temperature for 24h. Based on our previous studies [17–19] we chose to use the heparin concentration of 20 µg/ml that is higher than the highest concentration of rh-chymase (10 µg/ml). Following incubation, the sections were fixed and stained using the direct IF staining protocol of fibrinogen at the pathology department of the hospital. Possible changes in IF staining of fibrinogen between these three groups were evaluated with a Leica fluorescence microscope.

The effect of rh-chymase pre-treatment on thrombin-induced clotting time of fibrinogen

At first 22 µl of rh-chymase (final concentration 0, 0.1, 1.0 and 10 µg/ml) was mixed with 403 µl of 100 mM Tris-HCl buffer (pH 7.6), 50 µl of 9 % NaCl (10x D-PBS), 5 µl of 1 M $CaCl_2$, 10 µl of 50 mg/ml fibrinogen (final concentration 1 mg/ml) and 10 µl of 1 mg/ml heparin (final concentration 20 µg/ml) or aqua. The mixture was incubated at 37 ºC for 30 min. Thrombin inactivation by mast cell chymase has been previously shown in rodents[20]. Therefore, we inactivated rh-chymase before adding thrombin by incubating the mixture with 5 µl of chymostatin (final concentration 20 µg/ml) for 10 minutes. To induce fibrin gel formation, the samples were mixed with 50 µl of thrombin (127 NIH Units/ml). The thrombin-induced clotting time was measured visually by gently mixing the solution continuously in a water bath at 37 ºC.[21]

The effect of rh-chymase-induced fibrinogen degradation products on the clotting time of human plasma

Nine blood samples from six healthy volunteers were added to vacuum tubes containing K_2EDTA. Plasma was separated by centrifugation. To make fibrinogen degradation products by rh-chymase, 1 mg/ml fibrinogen in PBS was incubated with rh-chymase (final concentration 0, 1 or 5 µg/ml) and heparin (final concentration 10 µg/ml) for 0 or 30 minutes at 37 ºC. Following incubation, rh-chymase was inactivated by incubating the mixture with chymostatin (final concentration 20 µg/ml) at 37 ºC for 30 min. Subsequently, 44 µl of this fibrinogen solution was combined with 40 µl of 200 mM $CaCl_2$ and 360 µl of EDTA-plasma which was mixed gently in a water bath at 37 ºC. The clotting time was measured visually.

The effect of rh-chymase on cross-linked fibrin

To produce cross-linked fibrin and fibrin gel, the following solution was mixed in wells of a 96-well plate; 160 µl of 100 mM Tris-HCl buffer (pH 7.6), 20 µl of 10x D-PBS, 10 µl of 200 mM $CaCl_2$, 10 µl of 20 mg/ml fibrinogen, and 20 µl of thrombin (127 NIH Units/ml). The gels formed were washed three times with PBS (10 min in each wash) whilst shaking. Thereafter, 200 µl of 0, 0.2, 2 or 20 µg/ml rh-chymase was added to the wells on the top of fibrin gels followed by incubation at 37 ºC for 2h or 24h. After incubation, the supernatant fluids from the wells were collected and the amount of residual gel in the well was estimated visually. To determine the amount of fibrin-derived protein products, the protein concentration in the collected fluids was analysed using the Pierce BCA protein assay (Thermo Fisher Scientific, Rockford, USA) and Tecan Sunrise Absorbance Reader at a wavelength of 570 nm. Bovine albumin was used as the standard.

The effect of rh-chymase on fibrin prepared from human plasma

Cross-linked fibrin was prepared from human plasma. For this experiment, blood samples from two healthy volunteers were added to vacuum tubes containing K_2EDTA, and the tubes were centrifuged to separate plasma. Subsequently, 180 µl of plasma from both volunteers was pipetted to eight wells of a 96-well plate and 20 µl of 200 mM $CaCl_2$ was added to each well to produce fibrin gel. After 30-min incubation, the fluid phase was removed, the gels were extensively washed, and treated with rh-chymase, as described above.

Statistical analysis

A two-tailed Wilcoxon Signed Rank test was used with IBM SPSS Statistics version 23 to evaluate the differences in percentages of chymase$^+$ mast cells in close association with fibrin$^+$ vessels between healthy skin, IP lesion and PP lesion. This test was also used when the differences in IF staining positivity of fibrin were assessed between the cryosections incubated with PBS, rh-chymase alone or rh-chymase in combination with heparin. P-values < 0.05 were considered statistically significant.

Results

Chymase-positive mast cells are in close association with fibrin-positive vessels in vasculitis cryosections

The percentage of chymase$^+$ mast cells in close association with fibrin$^+$ vessels was 5.0 ± 6.2 % (mean ± SD), 7.4 ± 8.1% and 5.0 ± 6.5% in the healthy skin, IP lesion and PP lesion, respectively (Fig. 1). The values were nearly the same at all stages of vasculitis and the differences between IP and heathy skin or PP and heathy skin were not statistically significant (Wilcoxon Signed Rank test).

Fibrinogen is degraded by rh-chymase and the reaction is enhanced by heparin

Purified human fibrinogen with 20 μg/ml heparin produced SDS-PAGE bands with calculated molecular sizes of 78 kDa, 68 kDa and 59 kDa corresponding to alpha-, beta- and gamma-chains of fibrinogen, respectively (Fig. 2). The treatment of fibrinogen with only 1 μg/ml rh-chymase already led to complete disappearance of the alpha-chain band with multiple cleavage fragments. Incubation with 10 μg/ml rh-chymase revealed some apparent cleavage of the beta-chain resulting in a somewhat smaller, 55-kDa band. Heparin enhanced the degradative effect of rh-chymase on the beta-chain. The beta-chain band almost disappeared already at a concentration of 1 μg/ml rh-chymase and 20 μg/ml heparin, and degraded completely with 10 μg/ml rh-chymase and heparin.

The gamma-chain was cleaved partially with 1 μg/ml rh-chymase and completely with 10 μg/ml rh-chymase. In addition, several low-molecular size bands were produced with 38 kDa, 35 kDa, 33 kDa and 17 kDa of Mr, as a result of alpha-, beta- or gamma-chain degradation. However, the band with a molecular size of the gamma-chain was stronger in the presence than in the absence of heparin at both 1 and 10 μg/ml rh-chymase. Heparin alone had no effect on fibrinogen.

The immunofluorescence staining of fibrin decreased after pre-treatment with rh-chymase and heparin enhanced the effect

The IF staining positivity was analysed semiquantitatively using a scale from 0 (negative) to 3 (strong). In all 10 samples, the IF staining was strong in vessel walls after PBS-treatment. In 7 of 10 samples, pre-treatment with 10 μg/ml rh-chymase decreased IF staining in vessel walls. Pre-treatment with rh-chymase and 20 μg/ml heparin further decreased the IF positivity in 6 of 10 samples. The staining positivity of fibrin was significantly decreased in cryosections treated with rh-chymase alone (mean 1.8, $p = 0.014$, Wilcoxon Signed Rank test) or with rh-chymase and heparin (mean 1.35, $p = 0.016$), compared to cryosections treated with PBS only (mean 2.4) (Fig. 3). In some samples, the IF positivity was very strong and could not only be seen in vessel walls, but also elsewhere in the dermis. In these samples, rh-chymase pre-treatment reduced IF positivity from the dermis, and rh-chymase together with heparin also decreased IF positivity from vessel walls.

Thrombin-induced clotting time was prolonged after pre-treatment of fibrinogen with rh-chymase

The experiment was repeated three times with very similar results (Fig. 4). Rh-chymase pre-treatment prolonged the clotting time in a dose-dependent manner with or without heparin in a similar fashion. Remarkably, no clotting was observed during the measurement period for up to 180 s after using 10 μg/ml rh-chymase.

Rh-chymase-induced fibrinogen degradation products increased the clotting time of human plasma

The $CaCl_2$-induced clotting time of EDTA-plasma was 8.51 ± 2.55 min, 7.35 ± 0.85 min and 8.37 ± 1.62 min by the fibrinogen solution pre-incubated for 0 min in the presence of 0, 1 and 5 μg/ml rh-chymase, respectively. However, after pre-incubation of fibrinogen with 0, 1 and 5 μg/ml rh-chymase for 30 min, the $CaCl_2$-induced clotting time of EDTA-plasma was 7.93 ± 1.70 min, 8.54 ± 2.11 min and 9.97 ± 2.76 min, respectively. In all 9 plasma samples, the clotting time increased by pre-treatment of fibrinogen with either 1 or 5 μg/ml rh-chymase. In 2 samples, the clotting time first increased by 1 μg/ml and then decreased by 5 μg/ml rh-chymase pre-treatment.

Rh-chymase degraded cross-linked fibrin

After 2-h incubation with rh-chymase, the residual fibrin gel was visually judged to be smaller in wells containing the highest rh-chymase concentration (10 μg/ml). After 24-h incubation, the fibrin gel totally disappeared at the highest rh-chymase concentration, and was smaller in size at 1 μg/ml rh-chymase than at 0.1 μg/ml or 0 μg/ml chymase. After 2-h incubation, the protein concentration in fluid samples was 434, 389, 414, and 482 μg/ml at 0, 0.1, 1.0 and 10 μg/ml rh-chymase, respectively, and after 24-h incubation 403, 672, 798, and 790 μg/ml, respectively. In other words, protein concentration increased with increasing concentrations of rh-chymase (Fig. 5). Rh-chymase protein alone did not explain these changes in soluble protein. The experiment was repeated twice with very similar results.

Rh-chymase degraded fibrin gel prepared from human plasma

After 2-h incubation with rh-chymase, the visual appearance of the fibrin gels did not change at any rh-chymase concentrations. After 24-h incubation at 10 μg/ml rh-chymase, the fibrin gel from the first donor disappeared fully, while the fibrin gel from the other donor was partially decreased in size and appeared to have a porous structure. The fibrin gels at 0, 0.1 and 1.0 μg/ml rh-chymase were stable visually. The protein concentration in the fluid increased slightly with increasing rh-chymase concentrations, but very strongly at the highest rh-chymase concentration after 24-h incubation (Fig. 6). The results of the two healthy plasma donors were very similar.

Discussion

Our present study demonstrates that rh-chymase degrades fibrinogen and cross-linked fibrin and prolongs clotting time. Rh-chymase degraded the alpha-, beta- and gamma-chains of fibrinogen, as detected in SDS-PAGE. Heparin alone had no effect on fibrinogen but it enhanced the degradative action of rh-chymase on the beta-chain of fibrinogen. We found some cleavage of the gamma-chain of fibrinogen with 1 μg/ml rh-chymase and complete cleavage with 10 μg/ml rh-chymase. However, a strong band with a molecular size corresponding to gamma-chain was seen in the presence of heparin at both rh-chymase concentrations. Heparin possibly prevents rh-chymase from degrading the gamma-chain, or alternatively, it promotes the generation of a new band from the beta-chain with nearly the same molecular size as that of the gamma-chain.

Fibrinogen pre-treatment with 0, 0.1 or 1 μg/ml rh-chymase prolonged the thrombin-induced clotting time in a dose-dependent manner. The addition of heparin did not affect the clotting time essentially, probably because rh-chymase had already efficiently degraded the alpha-chain of fibrinogen without heparin. When thrombin reacts with fibrinogen, it first degrades fibrinopeptide A from the alpha-chain of fibrinogen at α-Arg16, and subsequently, fibrinopeptide B from the beta-chain at β-Arg14.[11,12] Human chymase is known to cleave peptides most often after aromatic amino acid residues, especially phenylalanine and tyrosine.[22] It seems that the effects of rh-chymase on clotting are more versatile than just fibrinogen degradation. We demonstrated that rh-chymase-induced degradation products of fibrinogen increased the clotting time of plasma. Further studies are needed to identify the exact fibrinogen cleavage sites hydrolyzed by chymase and to examine the structure and functional role of these degradation products. An alternative explanation is that the degradation products of fibrinogen are peptides that simply act as competitive inhibitors or substrates for blood clotting enzymes.

Rh-chymase clearly degraded the fibrin gel prepared from pure fibrinogen more efficiently than the gel prepared from human plasma. The concentration of fibrinogen in plasma is normally 2-4 mg/ml, but it can multiply in many pathological conditions, such as inflammation.[23] In this experiment, the smaller fibrinogen concentration (1 mg/ml) in the gel prepared from purified fibrinogen possibly makes it more susceptible to degradation than the gel prepared from human plasma. However, as expected, the amount of protein degradation products in the fluid after the rh-chymase treatment was considerably higher in gels prepared from human plasma than in gels prepared from pure fibrinogen. Chymase is sensitive to the inhibitory effects of plasma protease inhibitors, including α_1-proteinase inhibitor, α_1-antichymotrypsin and α_2-macroglobulin[24,25]; this may explain the finding that the fibrin gel prepared from human plasma was clearly less sensitive to the degradation by rh-chymase than the gel prepared from pure fibrinogen. Plasma also contains plenty of different proteins, like albumin, which may be a competitive substrate for rh-chymase.[26]

The strong fibrinogen IF positivity decreased in most of the sections after pre-treatment with 10 µg/ml rh-chymase and decreased even further when pre-treated with rh-chymase and heparin. This finding parallels the results in SDS-PAGE and further suggests that chymase, known to degrade tissues and basement membrane zone[15,17,27], can break down fibrin even in cutaneous vessel walls of vasculitis patients *in vivo* and heparin further enhances the effect of chymase. However, this phenomenon may not easily take place *in vivo* owing to plasma protease inhibitors. In fact, chymase was found to undergo partial inactivation in the PP lesion of vasculitis, possibly due to increased immunostaining of chymase inhibitor alpha-1-antichymotrypsin, which may partly explain the finding that the IF positivity of fibrin increased steadily during the progression of vasculitis.[9] Nevertheless, the results of this study suggest that chymase may be a previously unrecognized endogenous fibrinolytic enzyme with anticoagulative properties. It may also be involved in the clearance of fibrin from vessel walls in aged vasculitis lesions.

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Figure legends

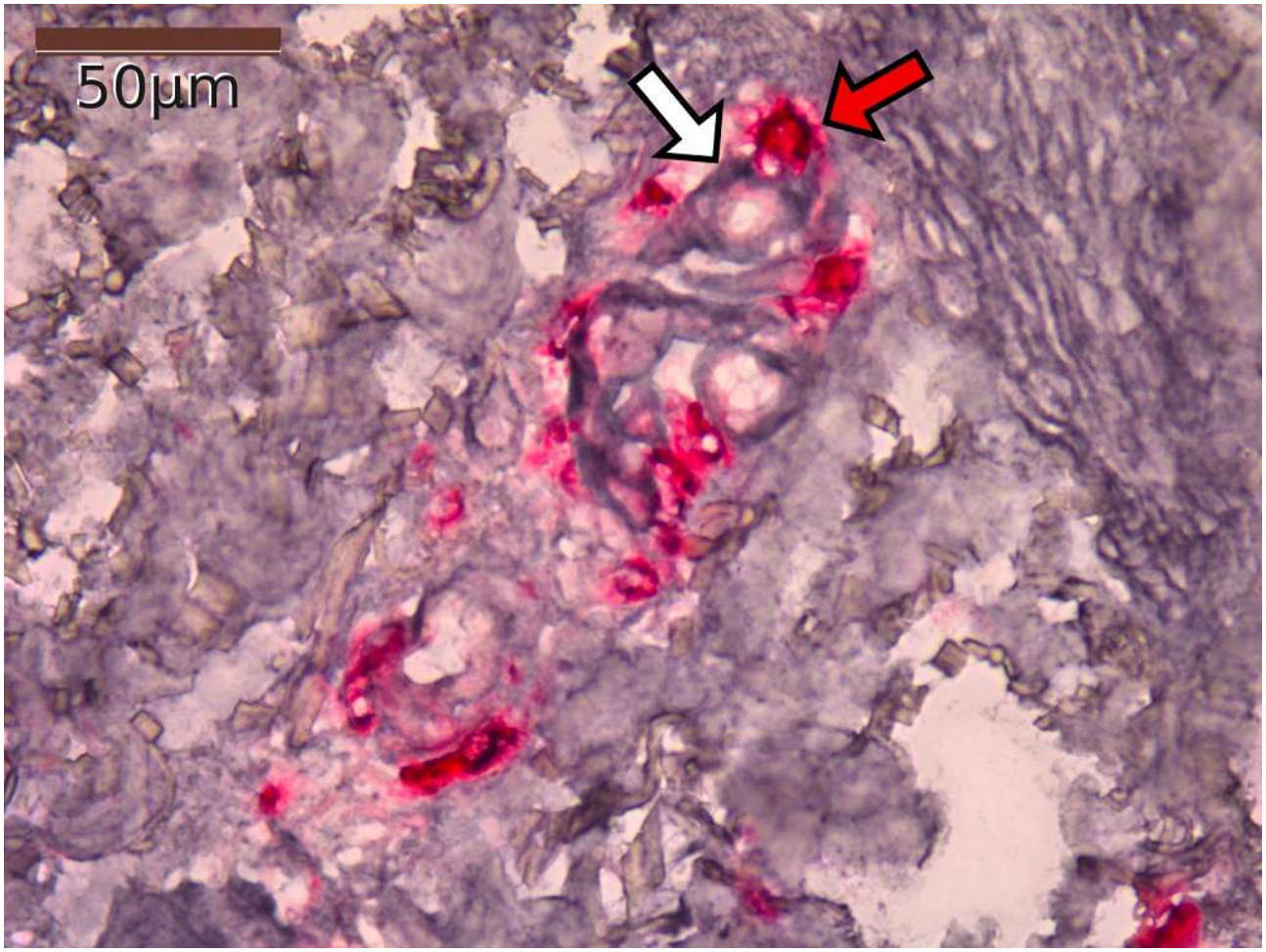

Figure 1. A sequential double-staining of mast cell chymase and fibrinogen on a cryosection of palpable purpura lesion of vasculitis. Chymase was stained immunohistochemically (red, red arrow). Subsequently, the same cryosection was stained immunohistochemically using polyclonal anti-human fibrinogen (dark, white arrow). Chymase^{+} mast cells and fibrinogen^{+} vessel walls are in close morphological contact. The background-like staining reflects the leakage of fibrinogen from vessels to dermis, which is typical in vasculitis inflammation.

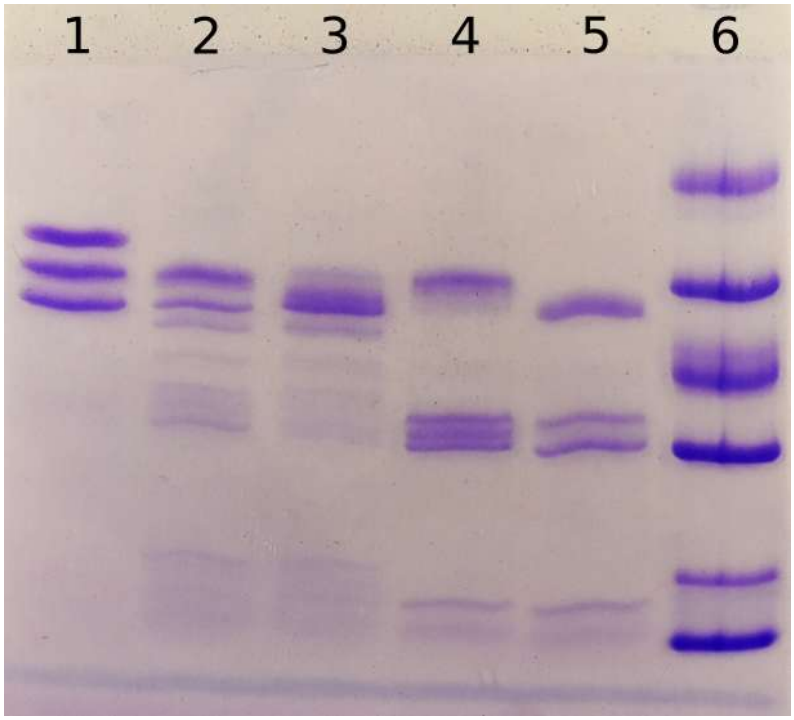

Figure 2. SDS-PAGE of human fibrinogen after incubation with rh-chymase. Fibrinogen (1.13 mg/ml) was incubated with 0, 1 or 10 µg/ml rh-chymase at 37 °C for 2h in the absence or presence of 20 µg/ml heparin followed by SDS-PAGE with 12.5% gel under reducing conditions. Lane 1: fibrinogen + heparin; lane 2: fibrinogen + 1 µg/ml rh-chymase; lane 3: fibrinogen + 1 µg/ml rh-chymase + heparin; lane 4: fibrinogen + 10 µg/ml rh-chymase; lane 5: fibrinogen + 10 µg/ml rh-chymase + heparin. Low molecular weight standards in lane 6 are phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

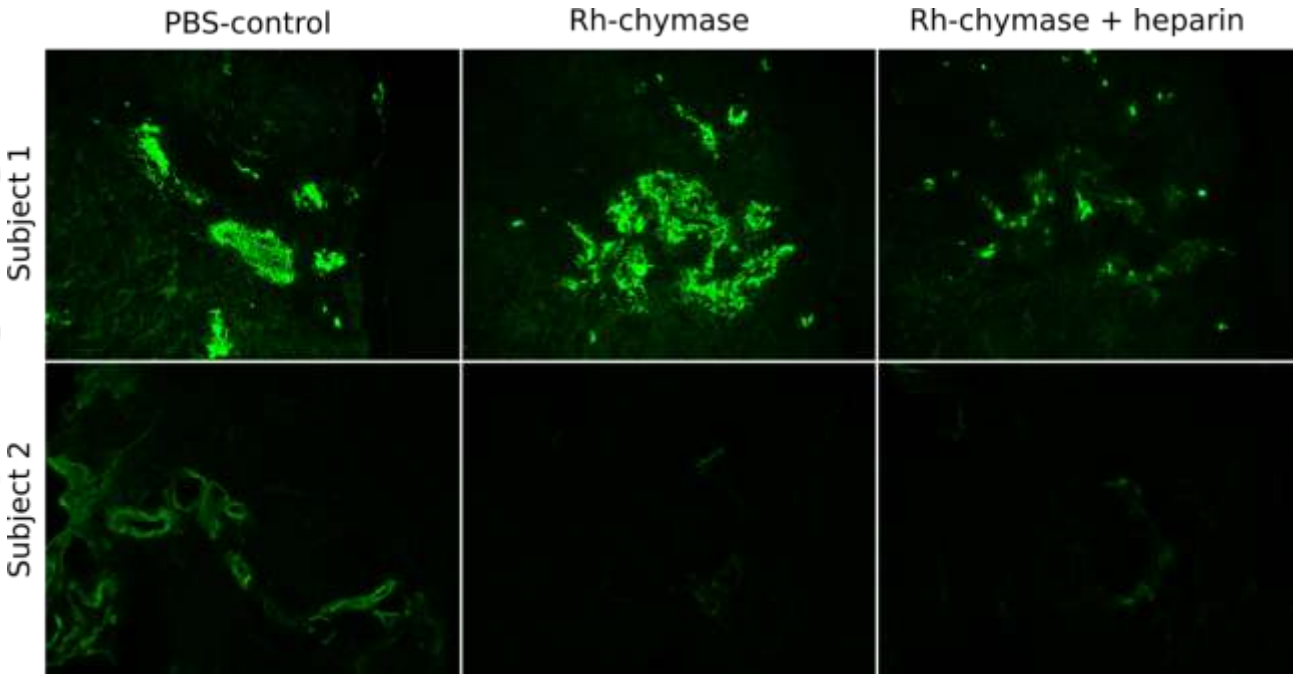

Figure 3. IF staining of fibrin for two representative subjects after 24-h incubation with diluent control (PBS), 10 µg/ml rh-chymase or 10 µg/ml rh-chymase and 20 µg/ml heparin. The IF staining was strong in vessel walls after PBS-treatment. Pre-treatment with 10 µg/ml rh-chymase decreased IF staining (subject 2) and pre-treatment with rh-chymase and 20 µg/ml heparin further decreased IF positivity (subject 1).

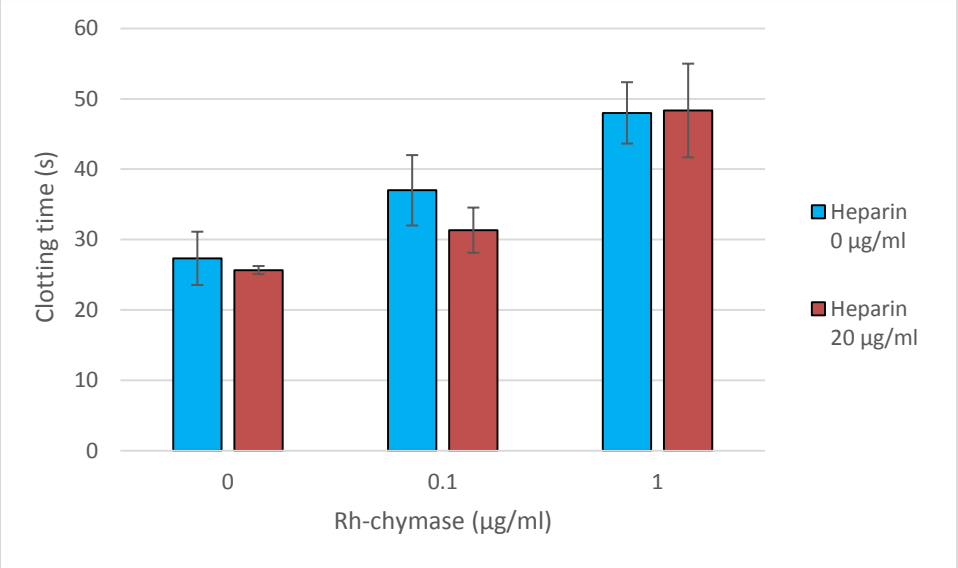

Figure 4. Thrombin-induced clotting time after rh-chymase pre-treatment of fibrinogen. Fibrinogen was pre-treated with rh-chymase (concentration 0, 0.1, 1.0 and 10 µg/ml) for 30 min in the absence or presence of heparin. The thrombin-induced clotting time was measured visually. The clotting time prolonged along with higher rh-chymase concentration in both incubation groups with (red columns) and without (blue columns) heparin in a similar fashion. With 10 µg/ml rh-chymase no clotting was observed during the follow-up for up to 180 s. The mean values and standard deviations of three equal experiments are illustrated in the histogram.

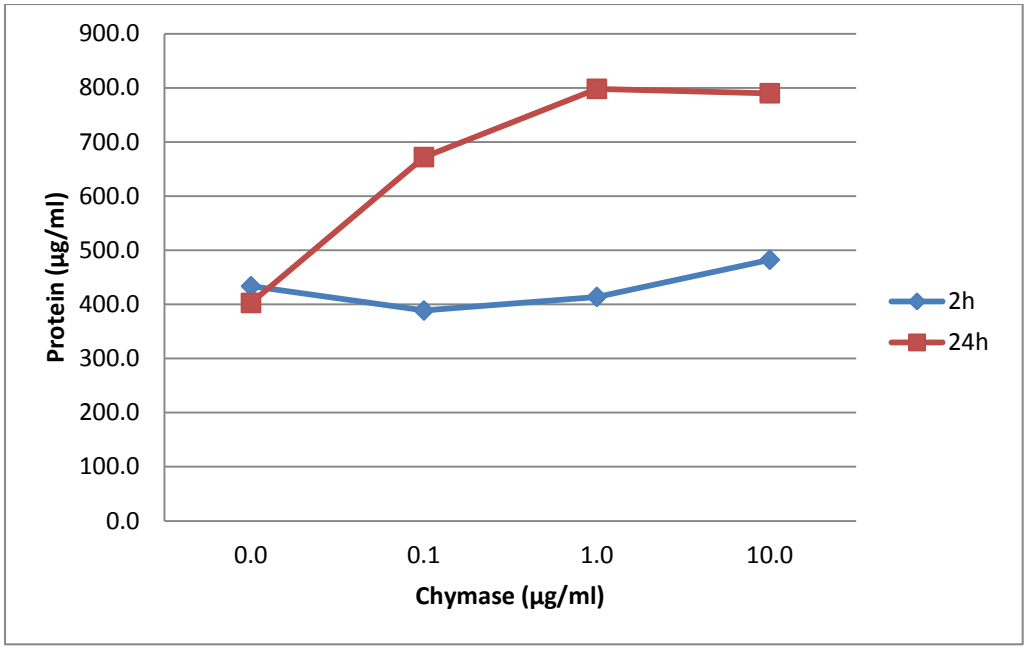

Figure 5. The degradative effect of rh-chymase on cross-linked fibrin. Cross-linked fibrin was incubated with rh-chymase (0, 0.1, 1.0 and 10 µg/ml) for 2 h or 24 h. Rh-chymase degraded cross-linked fibrin detected visually and released soluble protein from the gel when analysed by the Pierce BCA protein assay. The concentration of released degradation products increased along with increased rh-chymase concentration.

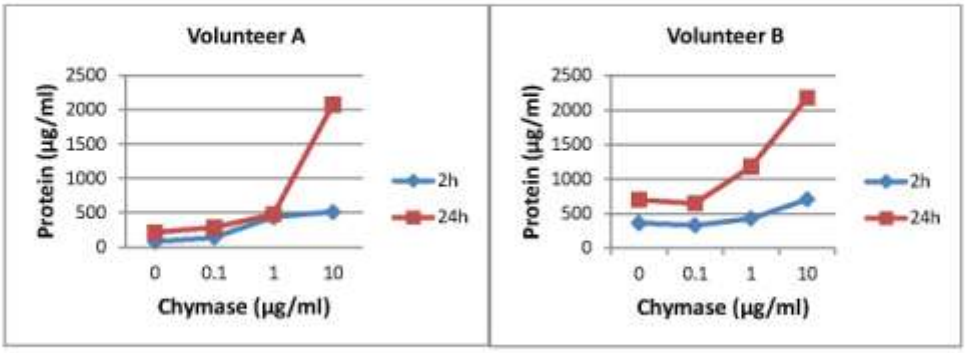

Figure 6. The degradative effect of rh-chymase on fibrin gels prepared from human plasma. Fibrin gels prepared from the plasma of two healthy volunteers were incubated with rh-chymase (0, 0.1, 1.0 and 10 µg/ml) for 2 h or 24 h. The degradative effect of rh-chymase on fibrin gel was analysed by detecting the concentration of soluble fibrin degradation products using the Pierce BCA protein assay. The protein concentration increased along with increased rh-chymase concentration. In volunteer A, rh-chymase degraded fibrin gel and released degradation products of fibrin notably with the strongest rh-chymase concentration and 24-h incubation. In volunteer B, the degradative effect was already remarkable at 1.0 µg/ml rh-chymase, but increased further with 10 µg/ml rh-chymase.