Acta Dermatovener (Stockholm) 51: 251–256, 1971

ELASTASE AND TRYPSIN INHIBITORS OF HUMAN SKIN AND SERUM

Partial Purification and Characterization

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Abstract. The elastase and trypsin inhibiting activity of human serum and skin homogenate was analysed. The homogenate of human skin was found to inhibit about one-tenth of the amount of elastase inhibited by the same amount of human serum. Fractionation of the skin homogenate by Sephadex gel filtration and ion exchange chromatography revealed one fraction capable of inhibiting both trypsin and elastase and one fraction which bound trypsin but not elastase. The first-mentioned inhibitor was found to bear a close resemblance to serum α_1-antitrypsin while the latter inhibitor of the skin could not be identified with any of the known serum trypsin inhibitors.

Human serum is known to contain inhibitors to elastase and trypsin. Elastase inhibitors have been reported to reside in the α_2-macroglobulin and 3.5 S α_1-glycoprotein (α_1-antitrypsin) fractions (2). The same serum fractions are known to contain the main part, over 90% of the serum antitryptic effect (10, 15). In these serum fractions, elastase and trypsin are bound in the same inhibitor proteins, which also are capable of binding chymotrypsin, thrombin, plasmin (3) as well as collagenase (4). Considerable changes in the levels of elastase and/or trypsin inhibitors have been reported to occur in several diseases, e.g. in familiar obstructive emphysema (5, 17), atherosclerosis (1), and elastosis cutis laxa (Ehlers-Danlos syndrome) (6).

Several tissues, other than serum, are known to contain their own characteristic proteinase inhibitors. Rat skin, e.g., contains an endogenous inhibitor for one of the proteinases of rat skin (9), and a proteinase and its specific inhibitor have been demonstrated in rabbit skin Arthus lesions (7). In surface washings of human skin, the occasional presence of elastase and/or elastase inhibitor has been claimed by Loeven (8).

In this paper we report our findings on trypsin and elastase inhibitors in human skin. The inhibitors of the same enzymes in human serum were analysed in parallel for comparison since most of the serum protein fractions are known to be present in the skin at high concentrations (13).

MATERIALS AND METHODS

Skin and serum samples

Skin samples were obtained from the operating theatre in connection with mastectomies or leg amputations. Several skin samples were combined, freed from subcutaneous fat, minced with scissors and homogenized with an Ultra-Turrax homogenizator in ten volumes of 10 mmol/l phosphate buffer, pH 8.0, containing, as a rule, 8% potassium chloride. The homogenate was centrifuged in an International Refrigerated Centrifuge at 8 000 *g* for 20 min. The supernatant was passed through a Millipore® filter (pore size 0.8 μm), and was used for further studies. All steps were performed at +4°C.

Samples of human serum were obtained by venepuncture from 20 young healthy adults, let to stand at room temperature for 60 min, and the clot was removed by centrifugation. A pooled preparation of the sera was used for characterization studies. Skin biopsies and serum samples from healthy skin areas of 20 dermatologic patients, affected by inflammatory diseases or psoriasis, were also collected and analysed individually. Homogenates from the skin biopsies were prepared as above, except that homogenization was carried out in 50 vol of the buffer.

Enzymes, substrates and buffers

Bovine pancreatic trypsin (Sigma, lot 58B-8000) was dissolved (5 mg/ml) in 0.025 N HCl containing 5 mmol/l $CaCl_2$. A 1 : 100–200 dilution in distilled water of the enzyme stock solution was used in the assays. Hog pancreatic elastase (Sigma, lot 78B-0670) was dissolved in distilled water (50–200 μg/ml). Bovine pancreatic α-chymotrypsin (Sigma lot 35B-2050) was dissolved in the same way as trypsin.

Remazolbrilliant Blue-elastin for elastase was prepared

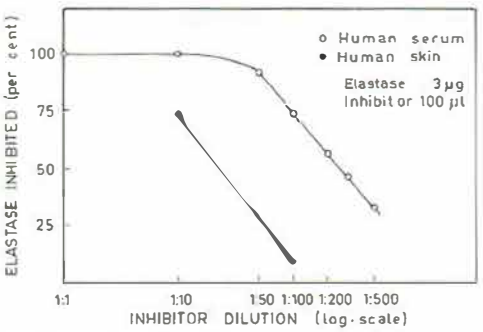

Fig. 1. The inhibition of elastase by human serum and skin homogenate at different dilutions. Assay conditions as given in Materials and Methods.

from elastin powder (Sigma, lot 29B-0950) and remazol-brilliant-Blue B (RBB, Farbwerke Hoechst A. G., Frankfurt) according to Rinderknecht et al. (12). N$^{\alpha}$-benzoyl-DL-arginine-*p*-nitroanilide (BAPA, Sigma) was dissolved in distilled water (1 mmol/l). Soybean trypsin inhibitor (SBTI) was obtained from Sigma Chem. Co. Tris-HCl buffer (0.1 mol/l, pH 7.8) was used in assays with trypsin and chymotrypsin, and Britton-Robinson universal buffer (11) pH 9.0 in assays with elastase.

Distribution of proteins was observed by measuring absorbancy at 280 nm and distribution of chloride by using the method of Schoenfeld & Lewellen (14).

Assay methods

For the measurement of elastase inhibition, the incubation medium consisted of 100 µl of the sample to be analysed, 200 µl of buffer solution and 100 µl of the enzyme solution. After a preincubation period of 5 min at 37°C, 3 mg of RBB elastin was added, and their mixture was incubated at 37°C for 1–6 h. The dilution of the enzyme solution was adjusted to allow a maximal inhibition of 60–80%. Reaction was terminated by adding 200 µl of a 10% solution of zinc sulphate. The tubes were centrifuged at 2 000 *g* for 15 min, and the amount of RBB liberated in the supernatant was measured with a Hitachi-Perkin Elmer UV-Vis spectrophotometer at 595 nm. Tubes in which the enzyme was added at the end of the incubation period were used as blanks. Tubes in which the inhibitor sample was replaced with a suitable buffer served as additional controls. The reaction was found to be linear in time under the conditions and incubation times used in the experiment. The inhibitor concentration was expressed as the amount (µg) of enzyme inhibited by 0.1 ml of the inhibitor solution. In the assays on the individual skin biopsies and serum samples, the amount of elastase was 3 µg per tube and the skin homogenates (1 : 50) were used undiluted while the serum samples were diluted 1 : 150 with the homogenization buffer.

In assays for trypsin inhibitor, the incubation medium consisted of 100 µl of suitably diluted inhibitor solution, 100 µl of the trypsin solution and 100 µl of buffer solution, which was preincubated at 37°C for 5 min. After addition of 100 µl of the BAPA solution, incubation was carried out at 37°C for 5–30 min. The dilution of the enzyme solution was adjusted to allow a maximal inhibition of 60–80%. The reaction was terminated by adding 200 µl of 1 mol/l acetate buffer, pH 4.2, containing 5% of phosphotungstic acid. After centrifugation at 2 000 *g* for 15 min, absorbancy was read with spectrophotometer at 383 nm. Blanks and controls were prepared as for assays for elastase inhibitor. The inhibitor concentration was expressed as in the case of elastase inhibitor.

The trypsin binding protein (TBP) is known to protect trypsin from the action of SBTI without markedly affecting the activity of the enzyme toward BAPA. Thus, assays for this protein were performed by allowing trypsin to preincubate at 37°C with the fractions to be analysed for 15 min, after which 100 µl of buffer containing SBTI (twice the amount of trypsin) was added. Substrate solution was added 15 min later and the mixture incubated for 15 min. The amount of TBP was expressed as the amount (µg) of non-STBI-inhibitable trypsin in assay. All of the numerical data reported are based on at least three independent determinations.

Chromatographic procedures

Gel chromatography was performed in a 10 × 100 cm column packed with Sephadex G-100 (Pharmacia, Uppsala) and equilibrated with 10 mmol/l Tris-HCl buffer, pH 7.0. The sample applied to the column was 240 ml of either skin homogenate or a 1 : 16 dilution of serum in 10 mmol/l phosphate buffer, pH 8.0. Equilibration buffer was used for elution, which was carried out as an upwards run by using a peristaltic pump. Fraction

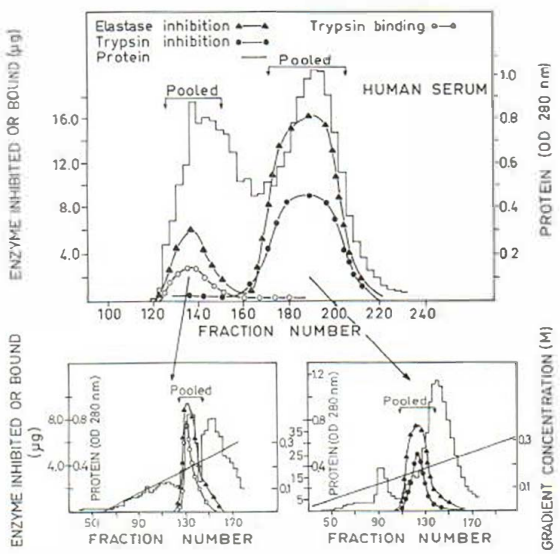

Fig. 2. Elastase and trypsin inhibition and trypsin binding by human serum fractions. Fractionation on Sephadex G-100 is shown in the upper part of the figure. Fractionation of the pooled preparations on DEAE-cellulose is shown in the lower parts of the figure. Assay conditions as given in Materials and Methods.

volume was 14 ml, temperature +4°C. The chromatographic fractions containing inhibitors were pooled and concentrated 4–8 folds using membrane ultrafiltration (Diaflo UM-10 and PM 30 membranes).

DEAE-cellulose chromatography was carried out in columns (2 × 25 cm) packed with microgranular DEAE-cellulose (Whatman DE 11), equilibrated with 10 mmol/l Tris-HCl buffer, pH 7.8. A linear gradient of NaCl (0–0.3 mol/l) in the above buffer was used for elution. The distribution of proteins and inhibitors was followed and the active fractions were pooled.

Characterization procedures

The pooled inhibitor preparations were dialysed against 10 mmol/l Tris-HCl buffer, pH 7.8 and protein concentration adjusted to 1 mg/ml by using the ultrafiltration device. Heat stability of the inhibitors was tested by exposing the samples of inhibitors to different temperatures in a water bath for 15 min. pH stability was tested by exposing the inhibitor samples to different pH-values (2.5–7.0) for 30 min at 37°C by adding 0.1 N HCl whereafter the samples were neutralized with 0.1 N NaOH and tested for inhibitor activity.

RESULTS

Elastase inhibition by serum and skin homogenate

The inhibition of elastase (3 μ per assay) by different dilutions of human serum and skin homogenate (0% in respect to KCl) is shown in Fig. 1. A 50% inhibition was obtained by using a serum dilution of 1 : 250 or a skin homogenate dilution of 1 : 25. Serum thus contained tenfold the amount of inhibitor present in an equal weight of human skin.

Fractionation of serum inhibitors

Gel chromatographic fractionation of serum proteins into two major protein fractions is presented in Fig. 2, upper part. Trypsin-binding protein was eluted in the first protein peak and trypsin inhibitor was present in the second. This chromatographic distribution is concordant with the well known fact that trypsin-binding protein is α_2-macroglobulin, while the trypsin inhibitor is α_1-globulin. The hydrolysis of RBB-elastin by elastase was inhibited by factors present in both of the protein peaks coincident with the distribution of proteins reacting with trypsin. This result could be anticipated on the basis of the known fact that α_2-macroglobulin as well as α_1-antitrypsin act as elastase inhibitors, too. On the basis of calculations on the size of the inhibitor peaks, it was estimated that about 10% of the total elastase inhibitor was present in the first peak and about 90% in the second.

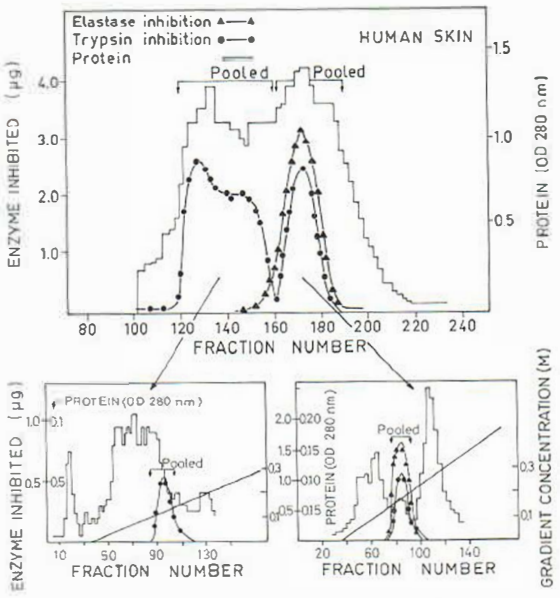

Fig. 3. Elastase and trypsin inhibition by the fractions of human skin. Fractionation of the homogenate on Sephadex G-100 is shown in the upper part of the figure. Further fractionation of the pooled preparations on DEAE-cellulose chromatography is shown in the lower parts of the figure. For details see text.

The fractions containing TBP and those containing trypsin inhibitor were pooled separately, as shown in Fig. 2, upper part. The preparations were concentrated 4- and 7.5-fold, respectively, and subjected to ion exchange chromatography on DEAE-cellulose. The results of the chromatographies are shown in Fig. 2, lower part. The TBP-preparation was fractionated into three main protein components. The second protein peak contained both the TBP and elastase inhibitor activities as a single peak (Fig. 2, lower left), eluted out at a 0.19 mol/l concentration of NaCl. The proteins of the trypsin inhibitor preparation, on the other hand, were eluted as two separate peaks. Trypsin and elastase inhibitor activities were eluted concomitantly, as a single peak, at a NaCl concentration of 0.18 mol/l, in the early fractions of the second protein peak.

Fractionation of skin inhibitors

Skin homogenate proteins were separated into two main peaks, the elution characteristics of which corresponded to those of the serum protein peaks (Fig. 3, upper part). As in the case of serum, the second protein peak contained inhibitor ac-

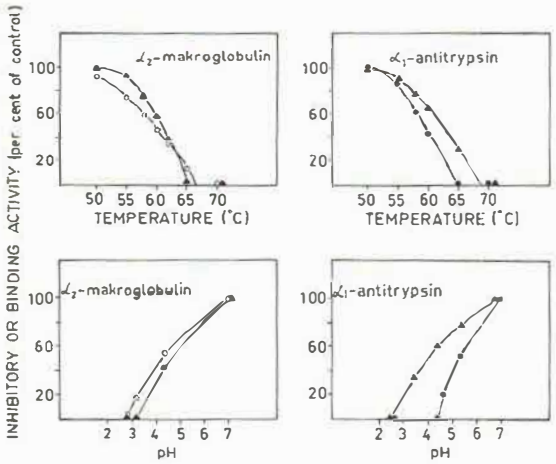

Fig. 4. The heat and pH stability of serum α_2-macroglobulin and α_1-antitrypsin. ▲—▲, elastase inhibition; ●—●, trypsin inhibition; ○—○, trypsin binding. For details see text.

tivity toward both trypsin and elastase, eluted in a single peak. The first protein peak, on the other hand, contained no elastase inhibitor and no TBP but, instead, a trypsin inhibitor.

Again, the fractions with inhibitor activity were pooled, concentrated, and subjected to ion exchange chromatography on DEAE cellulose. The results are given in Fig. 3, lower part. The trypsin inhibitor of the first preparation was eluted as a single peak at a NaCl concentration of 0.13 mol/l, in the later fractions of the main protein peak

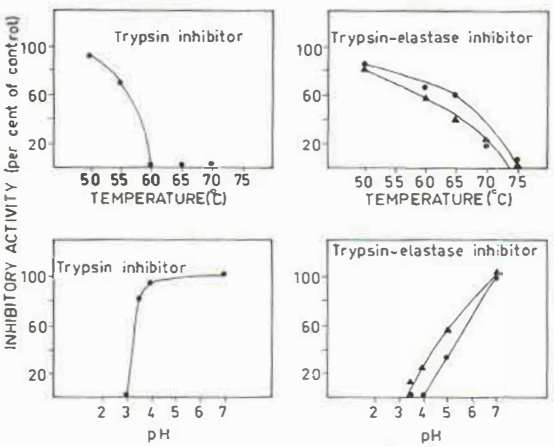

Fig. 5. The heat and pH stability of the skin trypsin inhibitor and of the skin trypsin–elastase inhibitor. ▲—▲, elastase inhibitor; ●—●, trypsin inhibition. For details see text.

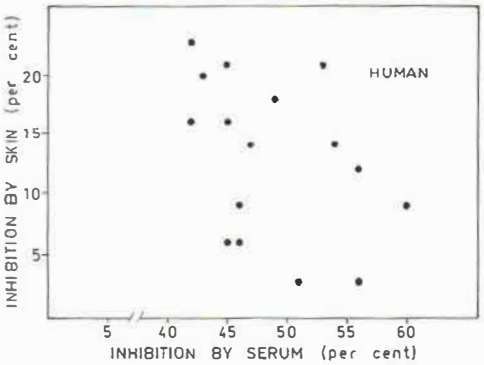

Fig. 6. The scattergram showing the correlation between elastase inhibition by human serum and skin. For details see text.

(Fig. 3, lower left). A considerable part of the inhibitor activity was lost, however, during the ion exchange chromatography, since only about 20 % was recovered in the fractions. Assays for elastase inhibitor and TBP were negative. DEAE-chromatography of the second inhibitor preparation showed a single peak with inhibitor activity toward elastase and trypsin, eluted at NaCl concentration of 0.17 mol/l, between two main protein peaks.

Stability characteristics

In tests for thermal and pH-stability, the trypsin-binding and elastase-inhibiting activities of the first serum peak, which contains α_2-macroglobulin, showed closely similar inactivation curves (Fig. 4, left part). The activities were fairly labile, showing a 50 % inactivation at 60–61 °C and at pH 4.3–4.6. The elastase-inhibiting and trypsin-inhibiting activities of the second serum peak, which contains α_1-antitrypsin, were also fairly labile, being inhibited to a 50 % level at 59–63 °C and at pH 4.0–5.3 (Fig. 4, right part).

The trypsin-inhibiting activity of the first peak of the skin chromatogram, on the other hand, was found to be quite labile to temperature, with a complete inactivation obtained at 60 °C. The pH-stability curve, on the other hand, showed essentially no inactivation at pH-values higher than 4, with a rapid decline in activity below this value (Fig. 5, left part). The stability curves for the inhibitor activities of the second skin chromatographic peak closely resembled those of the inhibitors of the second serum peak, with 50 % inactivation at 63–68 °C and at pH 4.8–5.5.

Correlation of serum and skin elastase-inhibitor levels

The scattergram in Fig. 6 presents data on the normal skin and serum elastase inhibitor levels in the patient material. No correlation between the inhibitor levels in the two tissues can be figured out. It was also impossible to relate any of the exceptionally high or low values to any specific dermatologic disease condition in this material.

DISCUSSION

The two distribution peaks of trypsin inhibition in the serum gel chromatogram are identifiable by their chromatographic behaviour and inhibitor characteristics as due to the presence of α_1-antitrypsin and trypsin-binding protein (α_2-macroglobulin). The chromatographic distribution curves of the two elastase inhibitors found in the serum coincide exactly with that of the trypsin inhibitors suggesting the same inhibitor proteins in both of the peaks to be inhibitory toward both trypsin and elastase. The concept that α_1-antitrypsin as well as α_2-macroglobulin could also inhibit elastase is supported by the conclusions of Baumstark (2) and Eisen et al. (4), based on different kinds of studies. In addition, the parallel destruction of the trypsin- and elastase-inhibiting capacities in our tests on temperature and pH-stability of the inhibitor preparations point to the same direction. In this study, the minor trypsin-inhibiting components of the human serum (10, 15) were not identified and, thus, it remains to be elicited whether these, too, are capable of inhibiting elastase.

The lower molecular weight protein fraction of the skin inhibited both trypsin and elastase. The identical chromatographic behaviour of these two inhibitor activities in both Sephadex and DEAE-cellulose runs suggests that they are due to the action of a single protein—a conclusion further supported by the stability tests. A comparison of the chromatographic and stability characteristics with those of serum α_1-antitrypsin discloses a marked similarity, and suggests these inhibitors to be identical. This view is supported by recent immunochemical findings on the presence of α_1-antitrypsin in the skin (13).

The higher molecular weight trypsin inhibitor of the skin appeared to differ markedly from the inhibitors discussed above, because of the lack of inhibition of elastase and, in addition, because of its greater pH-stability. The broad and biphasic elution curve in the Sephadex chromatography of this inhibitor could reflect the presence of two separate inhibitor proteins. The presence of one inhibitor peak, only, in the DEAE chromatogram would, in that case, signify that one of the inhibitors has been either retained in the column or inactivated during chromatography. The biphasicity of the Sephadex elution curve could alternatively be due to the fact that a strongly active BAPA hydrolysing trypsin-like enzyme, known to be present in the same fractions (16), rendered the inhibitor assays somewhat erroneous.

This inhibitor cannot yet be identified with any of the proteinase inhibitors reported to be present in the serum. The skin inhibitor described by Hayashi et al. (7) was reported to have a molecular weight of 12 500 and not to inhibit trypsin. It cannot thus be identical with any of the skin inhibitors described by us. The inhibitor described by Martin & Axelrod (9) in the skin was not described in such terms that definite conclusions on its similarity or dissimiliarity to any of our inhibitors could be made. Preliminary findings in our laboratory, however, suggest that some of the characteristics of this inhibitor resemble those of the skin elastase–trypsin inhibitor (to be published).

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Received November 2, 1970

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