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THE OCCURRENCE OF COMMON INHIBITORS OF TRYPSIN AND OF LEUCOCYTE NEUTRAL PROTEINASE IN HUMAN SERUM

A. KOJ, J. CHUDZIK, W. PAJDAK[a] AND A. DUBIN

Department of Animal Biochemistry, Institute of Molecular Biology, Jagiellonian University, and [a]Department of Clinical Chemistry, Institute of Internal Medicine, Medical Academy, Krakow (Poland)

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SUMMARY

1. Digestion of casein by commercial trypsin (EC 3.4.4.4), or by an extract of granules isolated from horse blood leucocytes was inhibited by human serum.

2. Human serum subjected to affinity chromatography on a trypsin–Sepharose column lost both trypsin and leucocyte neutral proteinase inhibiting capacities.

3. After reaction of serum inhibitors with an excess of either trypsin or leucocyte proteinase the second of these enzymes added to the same sample showed unimpaired proteolytic activity. The coordinated proteolysis of casein by a mixture of trypsin and of leucocyte granule extract was observed.

4. Fractions of human serum separated by polyacrylamide disc electrophoresis or preparative starch block electrophoresis showed a similar profile of trypsin and leucocyte proteinase inhibition. The main inhibiting capacity was associated with albumin and postalbumin fractions corresponding to α_1-antitrypsin.

INTRODUCTION

Specific granules of the polymorphonuclear leucocytes contain a variety of lysosomal enzymes and basic proteins which are released in inflammatory and other pathologic states, bringing about tissue damage[1–5]. Neutral proteinase (or proteinases) present in these granules is able to digest several protein substrates[1,6–11] and probably is responsible for vascular injury or depletion of cartilage matrix[2,12]. For this reason studies on the inhibition of leucocyte proteinases are of considerable interest[13–17].

It has long been recognized that mammalian serum has the capacity to inhibit the enzymatic activity of different proteinases. Trypsin (EC 3.4.4.4) may be inhibited by several plasma proteins designated as α_1-antitrypsin (α_1AT), α_2-macro-globulin (α_2M), the inter-α-trypsin inhibitor, α_1-X-glycoprotein, α_2-antitrypsin[18–22]. α_1-Antitrypsin is responsible for approximately 80–90% of the total trypsin-inhibiting capacity of human serum, but it also shows affinity to other proteinases such as chymotrypsin (EC 3.4.4.5), some proteolytic enzymes from leucocytes[15], from *Aspergillus oryzae*[23] and *Bacillus subtilis*[24], as well as pancreatic and skin elastases (EC

3.4.4.7)[25,26], plasmin (EC 3.4.4.14) and thrombin (EC 3.4.4.13)[27,28]. α_2-Macroglobulin is a polyvalent inhibitor of proteolysis active against trypsin, chymotrypsin, thrombin, plasmin[29,30], elastase[31,32] and kallikrein (EC 3.4.4.21)[33].

A relationship between trypsin inhibitors and leucocyte neutral proteinase inhibitors in human serum has already been postulated[15] but remains incompletely explained. When investigating the properties of neutral proteinase from horse blood leucocyte granules[34], we also carried out some experiments aimed at the comparison of serum inhibitors of trypsin and of the leucocyte enzyme.

MATERIALS AND METHODS

Proteolytic enzymes

A solution of bovine trypsin (Schuchardt, München, 10 000 benzoyl-DL-arginine ethyl ester units per mg) containing 75 μg of trypsin per ml of 0.9% NaCl was prepared weekly and kept frozen until used.

The saline extract of specific granules from horse blood polymorphonuclear leucocytes served as the source of leucocyte neutral proteinase. The leucocytes were isolated from fresh citrate horse blood by a combined method of sedimentation and centrifugation, residual red cells being removed by a brief exposure to 0.2% NaCl. The leucocyte pellet was resuspended in 0.2 M sucrose solution containing 150 units of heparin (POLFA, Warsaw) per 1 ml. The disruption of leucocytes was accomplished by vigorous shaking of the suspension in a glass bottle, and the nuclei were centrifuged off at 800 $\times$ *g* according to Chodirker *et al.*[34]. The viscous supernatant was centrifuged for 30 min at 20 000 $\times$ *g* and the obtained pellet of crude leucocyte lysosomes was washed once with 0.3 M sucrose solution by centrifugation in similar conditions. Finally, the granules were suspended in 0.9% NaCl, frozen and thawed three times, then centrifuged at 105 000 $\times$ *g* for 30 min. The supernatant, faintly yellow-green in colour, was diluted with 0.9% NaCl to a concentration of approximately 1 mg of protein per ml and was used in the experiments on the inhibition of neutral proteinase by serum.

Estimation of proteolytic activity and serum inhibitors

0.2 ml of the enzyme solution in a test tube was mixed with 0.2 ml of 0.9% NaCl or 0.2 ml of a suitably diluted human serum. Then 0.3 ml of 0.1 M sodium phosphate buffer (pH 7.4) was added and the mixture left for 15 min at room temperature. After pipetting 1.8 ml of 5% casein (B.D.H., Poole) solution in 0.1 M sodium phosphate buffer the test tubes were incubated in a water bath at 37 °C for 60 min. The reaction was stopped by the addition of 4.5 ml 10% trichloroacetic acid. The protein precipitate was filtered off and the absorbance of the filtrate at 280 nm was determined. Blank samples contained all constituents, but trichloroacetic acid was added prior to incubation at 37 °C. Under these conditions, the net increase in the absorbance of incubated samples was within the range of 0.4–0.6. To obtain approx. 50% inhibition of proteolysis, normal human serum was diluted 20–30 times for leucocyte proteinase and 60–90 times for trypsin activity estimation.

For convenience it has been assumed that one unit of serum inhibitors corresponds to the amount of serum which, in the described conditions, causes a reduction of $\Delta A_{280\ nm}$ by 0.1 in samples incubated with trypsin or leucocyte proteinase.

Miscellaneous techniques

In order to obtain a sorption material for the removal of trypsin inhibitors from the serum, bovine trypsin was coupled to Sepharose 4B (generously supplied by Pharmacia Fine Chemicals, Uppsala) with cyanogen bromide as described by Axén and Ernback[35]: 150 mg of trypsin and 20 ml of Sepharose suspension were used. The material obtained was exhaustively washed with 0.05 M glycine buffer, pH 8.6, containing 1 M NaCl (Buffer A) followed by 0.05 M glycine buffer containing 6 M urea pH 10.6 (Buffer B), 0.05 M phosphate buffer pH 6.0 (Buffer C), and finally with 0.9% NaCl.

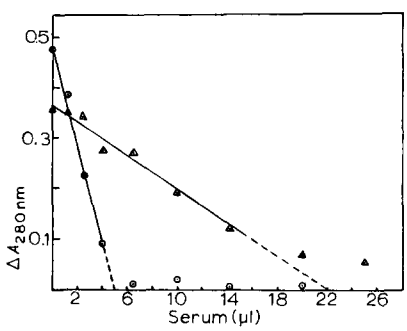
Polyacrylamide disc electrophoresis of human serum was carried out in vertical glass tubes in 7% gel, and starch gel electrophoresis on a horizontal block (30 cm × 15 cm × 1 cm) in Tris–citrate buffer, pH 8.6, as described by Gordon[36].

Protein contents in the leucocyte granule extract and in some serum fractions were determined by the method of Lowry *et al.*[37] with bovine serum albumin (Armour, Eastbourne) as the standard.

RESULTS

Inhibition of trypsin and leucocyte proteinase by human serum

When variable amounts of fresh human serum were added to bovine trypsin or horse leucocyte granule extract the digestion of casein by these enzymes was progressively inhibited as shown in Fig. 1.



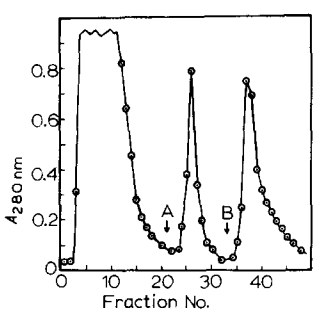


Fig. 1. Inhibition of trypsin and of leucocyte proteinase by human serum. 0.2 ml aliquots of fresh human serum progressively diluted with 0.9% NaCl were mixed with 0.2 ml trypsin solution or 0.2 ml leucocyte granule extract and incubated as described in Methods. The amount of serum added to the enzymes is given as µl of undiluted serum. $\Delta A_{280\,nm}$ corresponds to determined increases of the absorbance after 60 min incubation with the casein solution. ○, trypsin; △, leucocyte proteinase.

Fig. 2. Affinity chromatography of human serum on a trypsin–Sepharose column. A, 0.05 M glycine buffer, pH 8.6, containing 1 M NaCl; B, 0.05 M glycine buffer, pH 10.6, containing 6 M urea.

For trypsin a rectilinear relationship was obtained and it was established that 1 ml of serum contains 800–1000 units of inhibitors. As a rule, with leucocyte proteinase a higher scattering of experimental results occurred. The complete inhibition of the enzyme was not always observed, even at an excess of serum, this fact being reflected as "trailing" of the curve (Fig. 1). The slope of the curve was considerably

less steep than that of trypsin and it was calculated that 1 ml of human serum contains 140–170 units of the inhibitor.

Inhibition of elastolytic activity and haemoglobin-digesting activity of human leucocyte granule extract by human serum has already been observed by Janoff and Scherer[16], although the details of the reaction were not investigated. The phenomenon of inhibition of neutral proteinase from leucocytes by animal sera is rather common and shows no species specificity, as indicated by our experiments in which the enzymes from human or horse leucocytes were inhibited to a similar degree by human, horse, bovine and rat sera[38,39].

Affinity chromatography of human serum on a trypsin–Sepharose column

3 ml of fresh pooled human serum was diluted 10 times with 0.9% NaCl and applied to a trypsin–Sepharose column (1 cm × 12 cm) previously washed with suitable buffers (*cf.* Methods). A flow rate of approximately 12 ml/h was maintained with a peristaltic pump (UNIPAN). 3 ml fractions were collected and their absorbance at 280 nm measured (Fig. 2). After filtering of the diluted serum the column was washed with 30 ml of 0.9% NaCl. The absorbed proteins were then eluted with 30 ml of Buffer A followed by 30 ml of Buffer B. The obtained fractions were immediately neutralized by the addition of finely powdered NaH_2PO_4 and exhaustively dialyzed against a 0.9% NaCl solution. The samples containing proteins eluted with Buffers A and B were additionally concentrated 2–3 times by blowing air on the dialysing bag in a cold room. Finally, trypsin and leucocyte proteinase inhibiting capacities and protein contents were measured in the diluted serum and in the collected fractions.

It was found that the first protein fractions of human serum which filtered through the trypsin–Sepharose column are entirely devoid of trypsin and leucocyte proteinase inhibitors. Later the column becomes progressively saturated with inhibitors and they appear in the eluate. However, the ratio of specific activities of the inhibitors of both enzymes remains constant.

It was calculated that in the experiment shown in Fig. 2 over 90% of the total amount of trypsin inhibitors present in 3 ml of human serum was retained on the column. Attempts to recover these inhibitors by washing the column with alkaline buffers of high ionic strength or which contained urea were unsuccessful. The protein fractions eluted with Buffer A and B showed only weak trypsin inhibiting capacity (Samples 37 and 38, Fig. 2), or were totally inactive in the inhibition of casein digestion by trypsin and leucocyte proteinase. Polyacrylamide disc electrophoresis of these fractions revealed the presence of several proteins, mainly in the α_2- and γ-globulin region. Their identification by immunoelectrophoretic technique will be the subject of a separate study, but it appears that the native α_1-antitrypsin (the main serum trypsin inhibitor) is lacking in these fractions. It should also be mentioned that when the regenerated trypsin–Sepharose column was used for the second time, only a small proportion of trypsin inhibitors was adsorbed from the serum. This suggests that trypsin inhibitors of the serum are irreversibly bound to trypsin in the described conditions. In other experiments using freshly coupled trypsin–Sepharose, various buffers of the acid range (pH 4 and 6), or 0.1 M acetic acid, failed to elute substantial amounts of proteins after affinity chromatography of human serum.

Competition between trypsin and leucocyte proteinase for serum inhibitors

Assuming that serum contains common inhibitors of trypsin and leucocyte neutral proteinase it may be expected that after binding all inhibitors to either trypsin or proteinase the second of these enzymes added to the same sample will show unimpaired activity. To test this hypothesis the cross experiment was carried out: 0.2 ml of trypsin solution was mixed with 0.2 ml of human serum diluted 60 times and after 15 min 0.2 ml of leucocyte proteinase was pipetted followed by 1.8 ml of 5% casein solution. In another sample 0.2 ml of leucocyte proteinase was mixed with 0.2 ml of serum diluted 20 times, and after 15 min 0.2 ml of trypsin solution was pipetted followed by 1.8 ml of casein substrate. The remaining samples contained trypsin or leucocyte proteinase without or with diluted serum, or the mixture of the two enzymes as shown in the legend to Fig. 3. After incubation for 60 min all samples were deproteinized and the increase in the absorbance at 280 nm was determined.

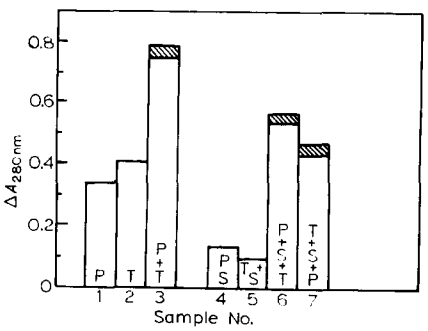


Fig. 3. Digestion of casein at pH 7.4 by trypsin and leucocyte proteinase, or a mixture of the two enzymes, in the presence or absence of diluted serum. P, leucocyte proteinase; T, trypsin; S, serum. Shaded areas show the increase of absorbance at 280 nm exceeding the expected value which had been calculated from the activity of individual enzymes.

The enhanced proteolysis of casein by the mixture of trypsin and leucocyte proteinase observed in samples 3, 6 and 7 arises from the fact that the two enzymes hydrolyze independently the substrate being in excess, and that they may split different polypeptide bonds, as suggested by Janoff and Zeligs[2].

Other data from this experiment indicate that binding of serum inhibitors by an excess of leucocyte proteinase (Sample 4) or trypsin (Sample 5) leads to the expression of full activity of the second enzyme added to the same sample. This observation suggests the identity of trypsin inhibitors and neutral proteinase inhibitors of human serum. Moreover, it may be supposed that the molecular sites of serum inhibitors binding trypsin or leucocyte proteinase are either identical, or of close spatial proximity, since binding one enzyme prevents the reaction of the inhibitor with the second proteinase.

Inhibition of proteolytic activities of trypsin and leucocyte neutral proteinase by fractions of human serum after gel electrophoresis

In preliminary experiments a sample of human serum was separated by polyacrylamide disc electrophoresis. One of the gel cylinders was stained for proteins with amido black while the other was sliced into 5 mm sections which were placed

in separate test tubes containing 0.5 ml of 0.05 M phosphate buffer pH 7.4. The test tubes were shaken for 12 h at 4 °C and then the trypsin (or leucocyte proteinase) inhibiting capacity of the buffer extract was determined with the casein substrate. A marked inhibition of both trypsin and leucocyte proteinase activities was produced by samples corresponding to albumin and postalbumin fractions and containing probably α_1-antitrypsin which migrates in this region. The distribution of trypsin and leucocyte proteinase inhibiting capacity of serum fractions in the gel was identical. No other serum fractions showed definite inhibitory properties but relatively small amounts of proteins could be separated by disc electrophoresis. For this reason in later experiments 0.15–0.45 ml of full serum was subjected to electrophoresis on a block of starch gel. After a 17 h run the block of gel was cut horizontally and one part stained for proteins with amido black. The second part was divided into 5 mm sections (perpendicularly to the long axis of the gel). Each section was immediately homogenized in a glass homogenizer with 2 ml of 0.1 M phosphate buffer pH 7.4. The homogenate was centrifuged at 2000 × *g* and 0.4–0.6 ml of the supernatant was used for estimating trypsin or leucocyte proteinase inhibition with 5% casein as the substrate. The results of these experiments are shown in Fig. 4.

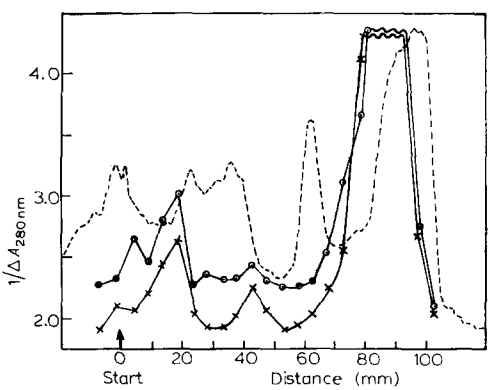


Fig. 4. Profile of inhibition of proteolytic activity of trypsin and leucocyte proteinase by human serum separated during starch-gel block electrophoresis. — — —, densitometric estimation of protein fractions on the starch block; ○—○, proteolytic activity of the leucocyte enzyme in the presence of corresponding serum fractions; ×—×, proteolytic activity of trypsin in the presence of corresponding serum fractions.

A major portion of the trypsin or proteinase inhibiting activity is again associated with the albumin and postalbumin fractions corresponding to α_1-antitrypsin. The second smaller peak visible near the start corresponds probably to α_2-macroglobulin, which is known to migrate slowly during starch gel electrophoresis. A third, very small peak occasionally noted in the intermediate fractions may represent inter-α- or α_2-trypsin inhibitors. However, at present no conclusion can be reached whether leucocyte proteinase is inhibited by some other proteins than those in the region of α_1-antitrypsin and α_2-macroglobulin. In general the two curves (for trypsin and leucocyte proteinase) show a striking similarity and almost coincide with each other. Our results with trypsin resemble the picture obtained by Kueppers[19], who employed the digestion of the fibrin agar plate after electrophoresis of human serum.

DISCUSSION

Fig. 1 shows that in order to obtain a comparable degree of inhibition 4 to 6 times more serum is required for the leucocyte proteinase than for trypsin. Since the molecular weights of both enzymes were found to be similar (J. Chudzik and A. Koj, unpublished results), the most likely explanation of the observed phenomenon is that leucocyte proteinase digests casein at a slower rate than trypsin. In such a case more enzyme is required to obtain the identical increase of absorbance at 280 nm, and proportionally more serum must be added to obtain similar inhibition.

The experiment depicted in Fig. 2 demonstrated that the insoluble derivative of trypsin efficiently removes both trypsin and leucocyte proteinase inhibitors from human serum. At the same time it is clear that affinity chromatography is not suitable for the isolation of the inhibitors from serum, in distinction to polypeptide trypsin inhibitors purified by this method by Fritz *et al.*[40]. At present it is difficult to decide whether this is caused by the formation of a stable covalent bond between the proteases and inhibitors, as suggested by the studies of Finkenstadt and Laskowski[41] and Ozawa and Laskowski[42], or whether the inhibitors were inactivated by a high pH or high urea concentration in the buffers used for elution.

The identity of serum inhibitors of trypsin and leucocyte neutral proteinase is strongly suggested by the results obtained independently with all the methods employed: affinity chromatography, competition for inhibitors, electrophoresis. It should be remembered, however, that these methods may be not sensitive enough to detect the presence of weak inhibitors constituting merely a few per cent of the total trypsin inhibiting capacity of human serum. Hence the final identification of individual serum inhibitors of leucocyte proteinase will be only possible with the employment of purified proteins.

Our results emphasize the biological function of the so-called serum trypsin inhibitors: they are probably involved in the protection of tissues against leucocyte proteinase which is released from white cells in various pathological states. Koj[39] observed that the leucocyte proteinase inhibiting capacity of rat serum was significantly increased 24 to 48 h after evoking aseptic inflammation in the rat. It is known that the α_1-antitrypsin level in plasma rises under a variety of pathologic conditions such as inflammation, malignant tumours and injection of typhoid vaccine while the serum concentration of α_2-macroglobulin does not change appreciably[19,22,43]. For this reason α_1-antitrypsin is regarded as a typical acute-phase reactant involved in the systemic response of the animal organism to unspecific trauma.

The importance of serum proteinase inhibitors is indicated by the fact that inborn deficiencies of α_1-antitrypsin are associated with the damage to lung tissue causing chronic obstructive emphysema[27]. It is supposed that proteolytic enzymes released from granulocytes in the course of local infection (bronchitis) destroy the lung tissue in the absence of proteolytic inhibitors of human serum[15]. Recently a case was reported linking α_1-antitrypsin deficiency with liver cirrhosis[44].

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