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THE IN VITRO INTERACTIONS OF RAT PANCREATIC ELASTASE AND NORMAL AND INFLAMMATORY RAT SERUM

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Summary

The partition of labelled rat pancreatic elastase (EC 3.4.21.11) between the different protease inhibitors of rat plasma was studied at different levels of saturation of the inhibitor capacity of plasma with the enzyme. The reaction mixtures were analysed by immunoelectrophoretic methods utilizing specific antisera against the different inhibitors and by gel filtration on Sephadex G-200. Rat serum was shown to contain four elastase binding proteins. α_1-antitrypsin, α_1-macroglobulin and α_2-acute phase protein and α_1-inhibitor$_3$ which exhibits immunologic cross-reaction with human inter-α-trypsin inhibitor and is of similar molecular weight. With minute amounts of labelled elastase the partition among the binding protein was α_1-macroglobulin 60%, α_1-antitrypsin 24% and α_1-I_3 16%. The 60% value of α_1-M bound radioactivity in normal serum corresponds to the sum of α_1-M and α_2-AP labelling in inflammatory serum.

Introduction

The elastolytic activity per gram tissue of rat pancreas is higher than that of other mammals [1]. We have recently isolated elastase (EC 3.4.21.11) from rat pancreatic juice and found it to possess a high specific elastolytic activity [2]. Studies of the interactions between pancreatic elastase and plasma protease inhibitors are few, especially those utilizing elastase and inhibitors from the same species.

Considerable attention has recently been devoted to the ability of elastase to hydrolyse elastin. This ability is unique among the pancreatic endopeptidases and may have a role in mediating tissue injury as indicated by the results of some reports [3,4,5]. The rat is a suitable experimental animal to investigate such mechanisms because of its high endogenous production of pancreatic elastase. A thorough elucidation of the interactions of this enzyme with the

protease inhibitors of rat serum is, however, a necessary prerequisite of such studies. A protein called α_2-acute phase globulin (α_2-AP) appears in rat serum in a high concentration after injury [6]. Since this inhibitor is homologous with human α_2-macroglobulin and with the normally present rat α_1-macroglobulin its appearance greatly influences the inhibitory capacity of rat serum. Moreover, a new proteinase inhibitor, recently isolated from rat serum which may be the homologue of the human inter-α-inhibitor, [7] was found to decrease after injury. Studies of normal as well as inflammatory rat serum were thus necessitated.

The purpose of the present investigation was to study the partition of labelled rat pancreatic elastase between the different protease inhibitors of rat serum at different levels of saturation of the inhibitory capacity of serum with the enzyme.

Materials

Rat serum was obtained by cardiac puncture under light ether anesthesia from normal and turpentine injected rats (strain: Sprague Dawley, 300—400 g bodyweight). Turpentine was injected subcutaneously (0.5 ml/100 g) 48 h before the blood sample was taken to get inflammatory rat serum.

Antibodies

Antibodies against each proteinase binding protein were obtained by injecting rabbits with purified antigens as described previously [8].

α_1-Macroglobulin and α_2-acute phase macroglobulin were purified earlier [8] and antibodies were available at the laboratory. α_1-I_3 was isolated as described in a previous paper [7] and α_1-antitrypsin was prepared according to the thiol Sepharose technique of Laurell [9] with two modifications: the material obtained from the thiol Sepharose column was dialyzed against 0.025 M phosphate buffer (pH 7.6) and chromatographed on a column (1.5 × 15 cm) of concanavalin A-Sepharose (Pharmacia) equilibrated with the same buffer. The concanavalin A bound material was eluted with 0.1 M α-methyl D-glucoside in the same buffer, concentrated by ultrafiltration (Diaflo membrane UM 10) and further purified on a Sephadex G-200 column (2.6 × 40 cm) under the same buffering conditions.

Elastase was isolated from rat pancreatic juice according to a previous report [2].

1 mg elastase in 1 ml 0.05 M sodium acetate buffer, (pH 6.0) 0.01 M $CaCl_2$ was labelled with ^{125}I according to the lactoperoxidase method of Thorell and Johansson [10]. Free iodine was eliminated by gel filtration on Sephadex G-50 (1.5 × 15 cm) equilibrated with 0.04 M Tris · HCl buffer (pH 7.6)/0.15 M NaCl/0.01 M $CaCl_2$. The specific activity was about 0.5 Ci/g protein . The labelled enzyme was stored at —40° C.

Radioactivity was counted in a well type scintillation detector.

Methods

5-μl aliquots fresh rat serum were mixed at room temperature with increasing amounts of ^{125}I-labelled elastase (1.25—11.5 μg active enzyme) and the

volumes were adjusted to 100 µl with 0.15 M NaCl. Samples were incubated for 15 min at room temperature before the analyses were started and could be used within 24 h provided they were kept at 4° C.

Crossed immunoelectrophoresis

Complexes between pancreatic rat elastase and serum inhibitors were demonstrated by crossed immunoelectrophoresis [11] in combination with autoradiography. Antisera against each proteinase inhibitor were mixed and added to the agarose gel for the second step. 6 samples containing increasing amounts of enzyme were analysed simultaneously with three specifically different antibodies.

Autoradiography was performed on the stained plate. The film (Kodak Tri X Pan) was exposed for 2 days.

Electroimmunoassay was performed according to Laurell [12]. The partition of ^{125}I-labelled elastase on the proteinase inhibitors was investigated as a function of enzyme concentration in normal an inflammatory serum. The influence of changed concentrations of proteinase inhibitors in turpentine injected rats, especially after the appearance of α_2-AP macroglobulins, was demonstrated by the relative avidity of each for elastase.

An agarose plate (21.5 × 11 cm) was divided in four parts with each containing specific antibodies (α_1-M, α_2-AP, α_1-AT and α_1-I_3). 5 µl of the different serum ^{125}I-labelled elastase mixtures were analysed simultaneously against these antibodies. Electrophoresis was performed for 15 h with a voltage of 4 V/cm. The plate was then washed 3 times with large volumes of 0.9% NaCl. The rocket antigen-antibody precipitates were then cut out and measured for their labelled enzyme contents in a gamma scintillation detector.

The binding of rat elastase to proteins of normal and inflammatory rat serum was also investigated using Sephadex G-200 chromatography.

125 µl normal or inflammatory serum were mixed at room temperature with 25 µl ^{125}I-labelled enzyme corresponding to about 7 µg enzyme. The volumes were then adjusted to 500 µl with 0.15 M NaCl. The mixture was then filtered through a column (2.6 × 45 cm) equilibrated in 0.05 M Tris · HCl buffer (pH 7.6)/0.5 M NaCl. 4.0-ml fractions were collected at a flow rate of 10 ml/h and analysed for absorbance at 280 nm and radioactivity.

Results and Discussion

Demonstration of complex formation between rat ^{125}I-labelled elastase and three proteins of normal rat serum

Reaction mixtures of serum and various amounts of enzyme were analysed by crossed immunoelectrophoresis. In binding the cathodal elastase, each protein demonstrated a reduced mobility on agarose gel electrophoresis. Two peaks, immunologically related, were demonstrated for each protein on antigen-antibody crossed electrophoresis (Fig. 1). The slowly migrating peaks contain the radioactivity by autoradiography and their relative size increases with the amount of added enzyme thus demonstrating that they contain complexes between the labelled elastase and the inhibitor. α_1-M and α_1-I_3 have about the same mobility on agarose gel at pH 8.6, whereas α_1-AT migrates

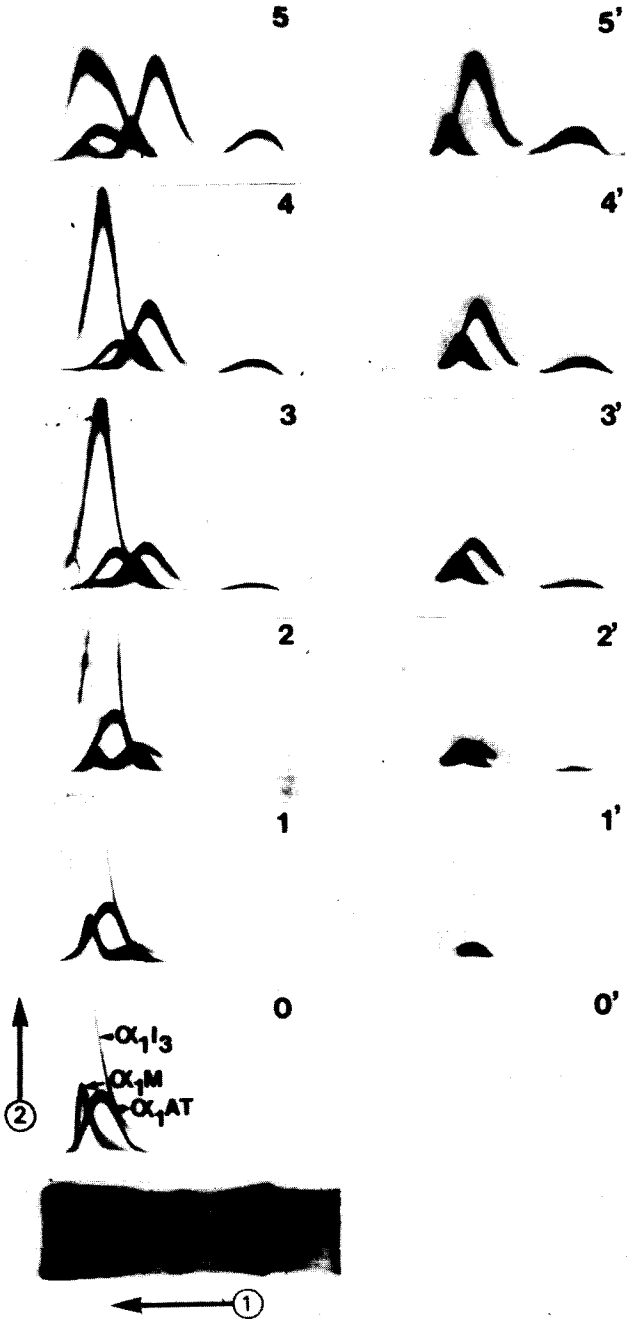

Fig. 1. Precipitation patterns obtained by antigen antibody crossed electrophoresis of rat serum reacted with increasing amounts of rat ^{125}I-labelled pancreatic elastase. For the second step the agarose gel contains a mixture of antibodies against α_1-M, α_1-I_3 and α_1-AT. 0 is the serum without enzyme, 1 to 5 the serum incubated with increasing amounts of enzyme. 0^1—5^1 are the corresponding autoradiographic pictures. The electrophoretic pattern of normal rat serum run simultaneously is given for reference.

more slowly. The first complex seen (Fig. 1, 1-1) was the one with α_1-M. Radioactive peaks with α_1-I_3 and α_1-AT begin to appear on autoradiography when about half the amount of α_1-M is complexed (Fig. 1, 2-2). Using inflammatory serum no modification of α_2-AP-mobility was seen after complex formation with elastase as demonstrated on autoradiography (Fig. 2).

Partition of increasing amounts of rat pancreatic elastase on proteinase binding proteins in normal and inflammatory rat serum

Reaction mixtures of serum and labelled enzyme were analysed by electroimmunoassay against specific antibodies and the antigen-antibody precipitates were measured for their radioactive content. Results are plotted in Fig. 3a for

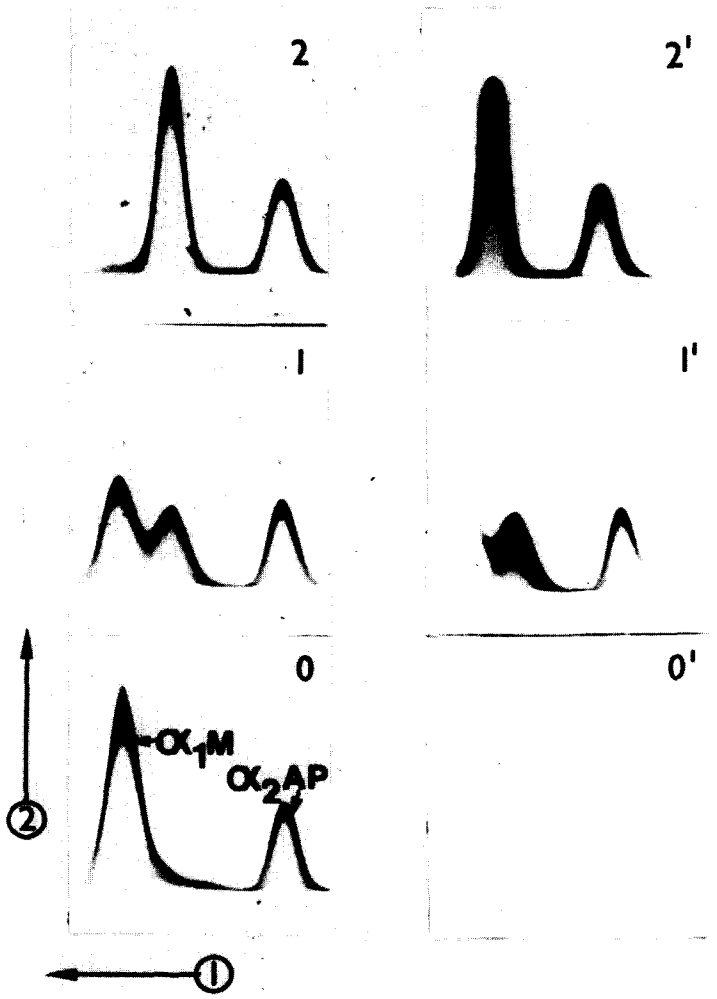

Fig. 2. Precipitation patterns obtained by crossed electrophoresis of inflammatory rat serum reacted with increasing amounts of rat ^{125}I-labelled pancreatic elastase. For the second step the agarose gel contains a mixture of antibodies against α_1-M and α_2-AP. 0 is the serum without enzyme, 1-2 the serum incubated with increasing amounts of elastase. 0^1—2^1 are the corresponding autoradiographic pictures.

each protein as a function of the enzyme amount added.

As in the previous experiment, pancreatic elastase was bound preferentially by α_1-M in normal rat serum. With minute amounts of labelled enzyme the partition with respect to the binding proteins was: α_1-M 60% ± 3%; α_1-AT 24% ± 3%; α_1-I_3 16% ± 3%.

The ratio of labelled enzyme complexed with α_1-AT and α_1-I_3 increases as α_1-M becomes saturated and reaches a plateau. The same result was obtained after the mixtures were kept at 4° C for 24 h. Consequently no significant transfer of

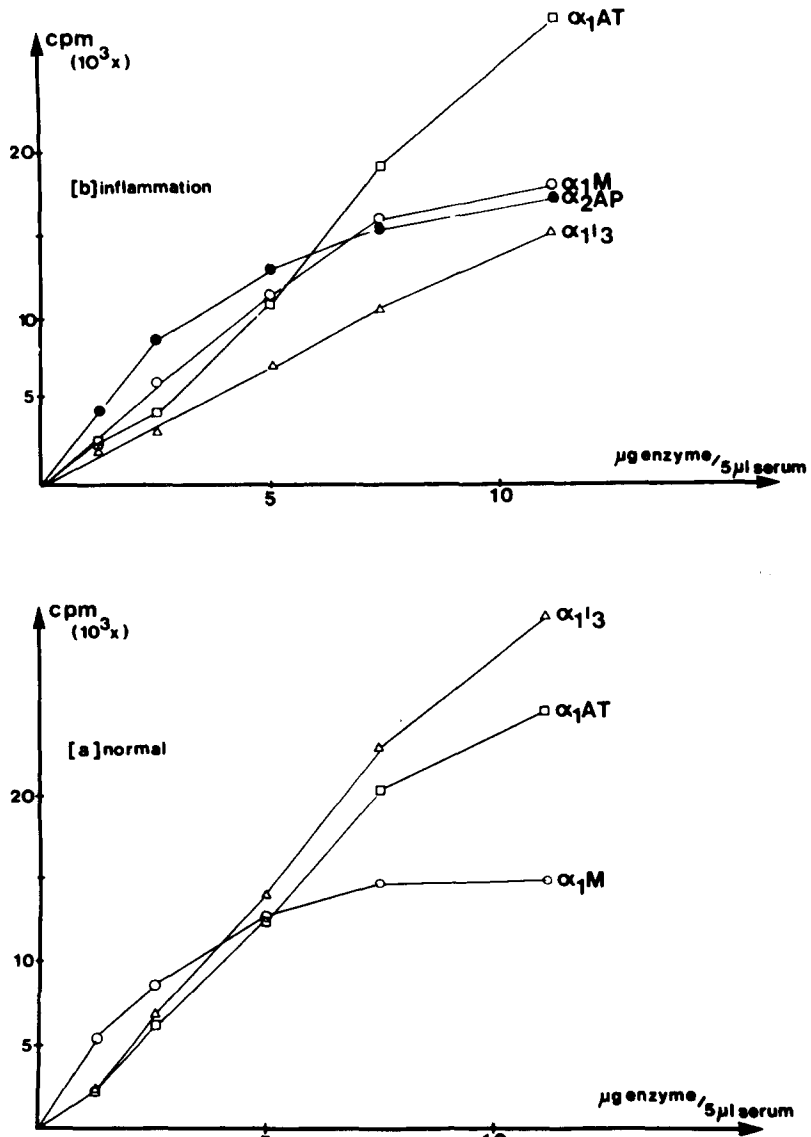

Fig. 3. Saturation curves of proteinase inhibitors from normal (a) and inflammatory (b) rat serum by ^{125}I-labelled pancreatic elastase. The results are expressed as the radioactivity content of the proteinase inhibitors-specific antibodies precipitates obtained by electroimmunoassay after reaction of constant amounts of serum with increasing amounts of labelled enzyme.

elastase from one inhibitor to another has occurred during that time contrary to the in vivo experiments carried out in human where a transfer from α_1-AT to α-M was observed [13]. This transfer could be related to the rapid elimination of α-M complexes in vivo favouring a shift towards the α-M.

The same experiment was performed with inflammatory serum [7] since the concentrations of proteinase inhibitors vary considerably during inflammation. α_2-AP which binds trypsin and chymotrypsin in the same way as α-M [8,14] appears under these conditions in variable amounts (2—9 g/l) [8] depending on the inflammatory response to the turpentine injection. We have, however, not found any significant change in α_1-M concentration which is about 3.7 g/l [15].

α_1-I_3 concentration in normal serum is about 2.5 g/l and during inflammation a decrease of about 60% was recorded after 48 h. On the other hand, a two-fold increase of the α_1-AT concentration was observed under the same conditions [7].

The distribution of increasing amounts of rat elastase among these four proteases is shown in Fig. 3. Because of the presence of α_2-AP macroglobulin, the percentage of elastase bound to α_1-M is decreased. This suggests competition between α_1-M and α_2-AP and indicates a rather similar mechanism of action of these two molecules as demonstrated previously using isolated molecules [14]. The 60% value of α_1-M-bound radioactivity in normal serum corresponds to the sum of α_1-M and α_2-AP labelling in inflammatory serum. In the result presented in Fig. 3b α_1-M and α_2-AP bind respectively 22 and 37% of the total bound radioactivity at a low concentration of enzyme. The percentage of α_2-AP-bound elastase is dependent on the concentration of this acute phase protein in serum. The amount of bound radioactivity is approximately constant at 60% when the enzyme bound to both macroglobulins is less than 1/3 of total saturation. This value of 60% remains constant independent of the α_2-AP concentration. α_2-AP seems to have a slightly higher avidity for elastase since it reaches its saturation plateau earlier. Changes in the enzyme binding of α_1-AT and α_1-I_3 are also observed in Fig. 3b. These variations are related to the modification of their concentration during acute inflammation [7].

Reaction mixtures of rat serum and rat pancreatic elastase were also studied by gel chromatography. This procedure permits study of the partition of labelled elastase among proteinase inhibitors with the ratio of enzyme to serum about 10-fold less than the lowest one used for electroimmunoassay.

This method, however, cannot discern partition of enzyme among each of the enzyme-binding proteins since α-M and α_1-I_3 are not separated well by Sephadex G-200. Only two peaks of radioactivity are eluted with the serum proteins (Fig. 4a). The first corresponds to complexes formed with α_1-M and α_1-I_3 and the second to the α_1-AT-elastase complex. A third peak is eluted later corresponding to unbound, inactive elastase. This peak represents 56% of the total radioactivity, and the 44% of total radioactivity bound to proteinase inhibitors agrees with the value previously found. The partition of enzyme also agrees with the previous result in normal serum that α_1-M and α_1-I_3 together bind 79% of the radioactivity, whereas α_1-AT binds 21%. In inflammatory serum (Fig. 4b) the first radioactive peak represents three different complexes (α_1-M, α_2-AP and α_1-I_3) and has nearly the same percentage of bound elastase as does normal serum (76%). This indicates again the competition between α_1-M

and α_2-AP. α_1-AT binds in this case 24% of the radioactivity and the same proportion of unbound enzyme was found in the third peak (Fig. 4).

The results of the present study show that rat serum contains four elastase binding proteins; α_1-AT, the two α-macroglobulins and a fourth protein (α_1-I_3) with some properties in common with human inter-α-trypsin inhibitor (ITI) [7]. Elastase is preferentially bound to the α-M compared to the other inhibitors. α_1-AT and α_1-I_3 show similar binding properties with rat pancreatic

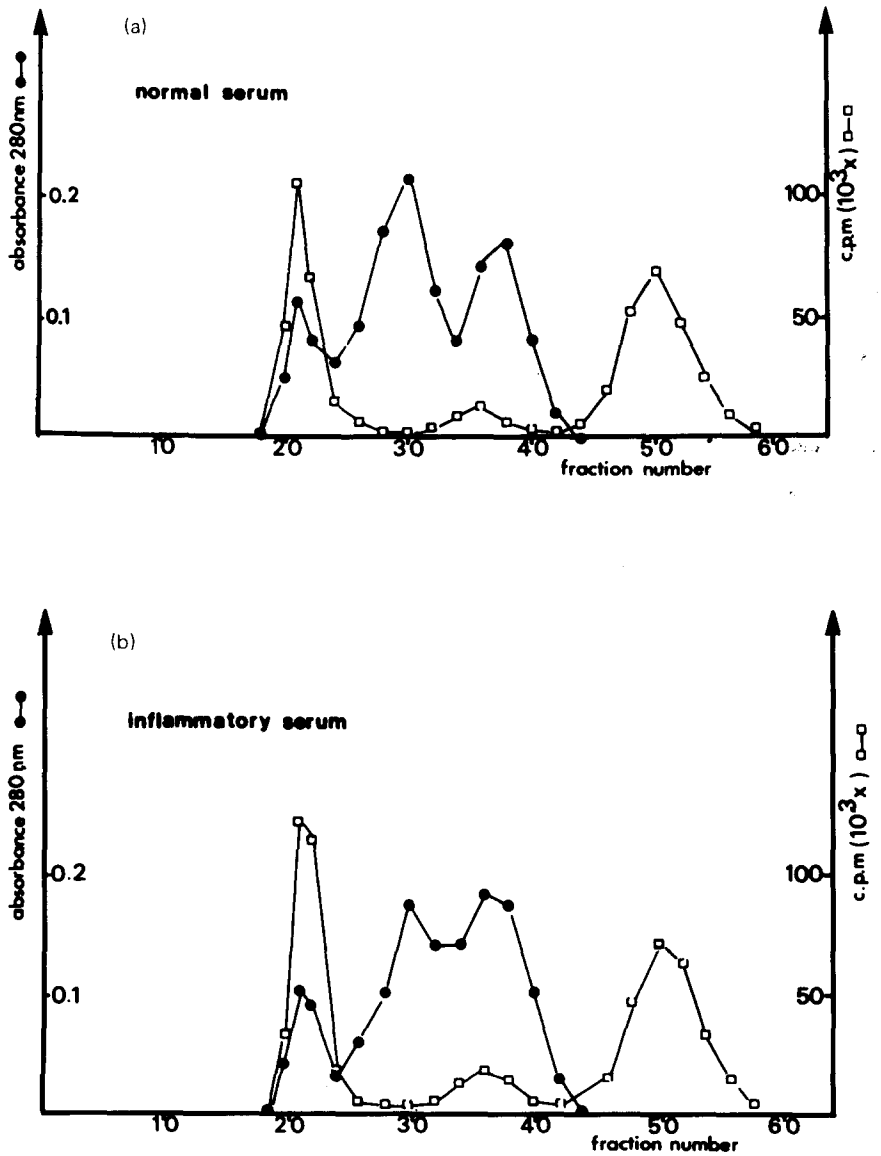

Fig. 4. Distribution of radioactivity (□———□) and proteins (●———●) in the fractions obtained by gel filtration on a Sephadex G-200 column (2.6 X 45 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.6, 0.5 M NaCl. Flow rate 10 ml/h. Fraction volume (4.0 ml) of a mixture of normal (a) and inflammatory (b) rat serum with minute amounts of ^{125}I-labelled elastase.

elastase. Katayama et al. [16], however, found a higher proportion of elastase eluted with the α_1-AT peak on Sephadex G-200. The fact they used an heterologous enzyme could explain this difference since it has been demonstrated in human serum, variations in the rate constants for the association of homologous and heterologous enzymes with α_1-AT [17]. The pronounced fall in the plasma concentration of α_1-I_3 in acute inflammation is of interest with regard to the biologic function of the inhibitor. Remarkably enough it was recently shown that newborn children with respiratory distress symptoms and infections had decreased plasma levels of ITI as compared to normals [18]. Further studies of the enzyme specificities of rat α_1-I_3 and its interactions with enzymes in vitro as well as further comparisons with the reactions of human ITI are under way in our laboratories.

Acknowledgements

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