Proteinase inhibitors in rat serum

Purification and partial characterization of three functionally distinct trypsin inhibitors

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Three different serine proteinase inhibitors were isolated from rat serum and purified to apparent homogeneity. One of the inhibitors appears to be homologous to α_1 -proteinase inhibitor isolated from man and other species, but the other two, designated rat proteinase inhibitor I and rat proteinase inhibitor II, seem to have no human counterpart. α_1 -Proteinase inhibitor (M_r 55000) inhibits trypsin, chymotrypsin and elastase, the three serine proteinases tested. Rat proteinase inhibitor I (M_r 66000) is active towards trypsin and chymotrypsin, but is inactive towards elastase. Rat proteinase inhibitor II (M_r 65000) is an effective inhibitor of trypsin only. Their contributions to the trypsin-inhibitory capacity of rat serum are about 68, 14 and 18% for α_1 -proteinase inhibitor, rat proteinase inhibitor I and rat proteinase inhibitor II respectively.

α_1 -PI is the major proteinase inhibitor in human plasma (Heimbürger, 1975). This protein has been shown to interfere with the action of a number of pancreatic serine proteinases, with leucocyte elastase probably representing the most important target (Morse, 1978). The observation that patients with severe deficiency in α_1 -PI have a predisposition to pulmonary emphysema (Kueppers & Black, 1974) has resulted in an increased interest in the properties and function of this protein.

α_1 -PI has been isolated and characterized not only from man (Pannell *et al.*, 1974) but also from plasma of several mammals, such as rabbit (Koj *et al.*, 1978), mouse (Takahara & Sinohara, 1982) and rat (Rosenberg *et al.*, 1976; Takahara *et al.*, 1980; Ikehara *et al.*, 1981; Roll & Glew, 1981; Thisner & Rosengren, 1982). Rosenberg *et al.* (1976) reported on two major α_1 -PI proteins in the rat. The two proteins were shown to differ in molecular size, to have different inhibitory specificities and to be immunologically distinct molecules.

Since work in our laboratory necessitated the isolation of pure α_1 -PI from small volumes of rat

serum (Dahlmann *et al.*, 1981), we used, in the present study, a new preparative ion-exchange medium (Duppel, 1982), together with two conventional steps, to purify α_1 -PI to homogeneity. In addition, two similar, but functionally distinct, proteinase inhibitors, tentatively designated RPI I and RPI II, were purified. RPI I appears to correspond to the R_2 component reported by Rosenberg *et al.* (1976), but RPI II represents a novel proteinase inhibitor that has not been previously identified in the rat.

Experimental

Materials

Male Wistar rats (Winkelmann, Borcheln, Germany) were used in all experiments. Serum was obtained from blood left at room temperature for spontaneous clotting.

Bovine trypsin (EC 3.4.21.4), bovine chymotrypsin (EC 3.4.21.1) and pig elastase (EC 3.4.21.11) were from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Bz-Arg-NPhNO₂ was from Merck, Darmstadt, Germany. Boc-Ala-Ala-Pro-Ala-NPhNO₂, Suc-Ala-Ala-Pro-Phe-NPhNO₂ and Serva Blue R as well as chemicals for polyacrylamide-gel electrophoresis were purchased from Serva Feinbiochemica, Heidelberg, Germany. 4-Nitrophenyl *p*-guanidinobenzoate hydrochloride, Bistris and Bistrispropane were

Abbreviations used: α_1 -PI, α_1 -proteinase inhibitor; RPI I and RPI II, rat proteinase inhibitors I and II respectively; Bz-, benzoyl-; Boc-, t-butoxycarbonyl-; Suc-, 3-carboxypropionyl-; -NPhNO₂, 4-nitroanilide; Bistris, 2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Bistrispropane, 1,3-bis-[(1,3-dihydroxy-2-hydroxymethyl)isopropylaminopropane].

from Sigma, München, Germany. Affi-gel Blue and protein standards for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories, München, Germany. Sephadex G-75 (superfine grade), heparin-Sepharose CL 6B, Blue Dextran 2000, DEAE-Sephacel and Mono Q anion-exchanger (Mono Q HR 5/5 prepacked column) were from Pharmacia, Uppsala, Sweden. Lab-Trol was from Dade, Miami, FA, U.S.A.

All other chemicals were of the highest purity available.

Assay of proteinase inhibitory activity

Anti-tryptic activity was determined with Bz-Arg-NPhNO₂ as substrate (Eriksson, 1965), antichymotryptic activity was determined with Suc-Ala-Ala-Pro-Phe-NPhNO₂ as substrate (DelMar *et al.*, 1979) and activity against elastase was measured with Boc-Ala-Ala-Pro-Ala-NPhNO₂ as substrate (Ashe & Zimmermann, 1977) by determining residual proteolytic activity after a preincubation of proteinase with inhibitor for 15 min at 25°C. Trypsin was purified from the commercial preparation of trypsin by the method of Jany *et al.* (1976), and the number of active sites in the trypsin preparation was determined to be 93% by active-site titration with 4-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967). The commercial preparations of chymotrypsin and elastase were estimated to contain 75% and 40% of active enzyme respectively. Appropriate corrections were made when calculating antiproteolytic activity.

One unit of inhibitory activity was defined as the amount of a preparation that completely inhibited 1 nmol of active proteinase. Specific activity was expressed as units of antiproteinase activity/mg of protein.

Protein determination

Total protein was determined by the method of Bradford (1976), with Lab-Trol as the standard.

Analytical polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out on linear-gradient 10–18%-acrylamide slab gels (Chua, 1980). Before electrophoresis, samples were treated as detailed elsewhere (Rutschmann *et al.*, 1982).

Chromatographic procedures

Affi-gel Blue chromatography was by a modification of a method described for fractionation of human plasma (Gianazzi & Arnaud, 1982). Ion-exchange chromatography on DEAE-Sephacel was carried out on a 2 cm × 20 cm column. The flow rate was 10 ml/cm² per h; 7 ml fractions were collected.

Chromatography on the Mono Q anion-exchanger was in conjunction with the FPLC (fast protein liquid chromatography) system (Pharmacia). Gel-exclusion chromatography on Sephadex G-75 (column dimensions 2.5 cm × 90 cm; flow rate 2 ml/cm² per h; fraction size 1.3 ml) was by the recycling technique (Porath & Bennis, 1962). All other experimental details were as given in legends to Figures.

Results

Purification of three trypsin inhibitors from rat serum

All steps were performed at 6°C, with the exception of Mono Q ion-exchange chromatography, which was performed at room temperature. Routinely, 20 ml samples were processed.

Step 1: chromatography on Affi-gel Blue. Serum was dialysed overnight against 25 mM-Bis-trispropane/HCl buffer, pH 6.7. A small turbidity that had formed during dialysis was removed by centrifugation (10000 *g* for 10 min), and the supernatant was fractionated on an Affi-gel Blue column. During this step, rat serum antitrypsin activity was resolved into three peaks (Fig. 1). The inhibitory-activity pattern towards trypsin, chymotrypsin and elastase is summarized in Table 1. Thus peak 1 material (α_1 -PI and RPI I) is active towards all three proteinases, peak 2 (RPI II) is inhibitory towards trypsin only, and peak 3 activity inhibits trypsin and chymotrypsin but not elastase. Since Affi-gel Blue chromatography of rat serum was analogous to the method described for human plasma (Gianazzi & Arnaud, 1982), peak 3 inhibitory activity was probably due to anti-thrombin III and α_1 -antichymotrypsin.

This was confirmed by the following experiment. Affinity chromatography of peak 3 material on heparin-Sepharose (Takahara & Sinohara, 1980) resulted in the selective adsorption of the antitryptic activity (antithrombin III), whereas the antichymotryptic activity (α_1 -antichymotrypsin) passed through the column unretarded. Importantly, under the same conditions, neither peak 1 nor peak 2 inhibitory activity was bound to the gel, showing that these two fractions were not contaminated by antithrombin III (results not shown).

Step 2: chromatography on DEAE-Sephacel (α_1 -PI and RPI I). The pooled peak 1 fractions from the Affi-gel Blue step, containing α_1 -PI and RPI I, were directly applied to a column of DEAE-Sephacel, equilibrated with 25 mM-Bis-trispropane/HCl buffer, pH 6.7. After passage of a small amount of unretarded protein, elution was performed with a linear gradient of 0–0.15 M-NaCl in the buffer (total volume 500 ml). Although no effective separation of α_1 -PI and RPI I is achieved during this step, a major fraction of inactive protein could

Fig. 1. Chromatography of rat serum on Affi-gel Blue

The column (3 cm $\times$ 42 cm) was equilibrated with 25 mM-Bistrispropane/HCl buffer, pH 6.7. After the sample (20 ml) was applied, the column was washed with 1200 ml of equilibration buffer, and elution was continued with equilibration buffer containing 1 M-NaCl and 0.5 M-KSCN successively. The flow rate was 8 ml/cm² per h, and 7.5 ml fractions were collected. —, A_{280} ; ●, trypsin-inhibitory activity.

Table 1. Proteinase-inhibitory activity of the eluate fractions from the chromatography of rat serum on Affi-gel Blue
For full experimental details see the text.

Peak no.	Proteinase-inhibitory activity (units/mg)		
	Antitrypsin	Antichymotrypsin	Antielastase
1	3.0	2.8	4.0
2	4.4	0	0
3	0.1	0.08	0

be removed, which facilitated the following step. The fractions with antitrypsin activity were pooled, concentrated to 32 ml by ultrafiltration and dialysed overnight against 20 mM-Bistris/HCl buffer, pH 6.0.

Step 3a: anion exchange chromatography on Mono Q (separation of α_1 -PI and RPI I). The dialysed sample from the DEAE-Sephacel step was applied to a Mono Q column, equilibrated with 20 mM-Bistris/HCl buffer, pH 6.0. Because of the limited capacity of the column (about 20 mg of protein), two successive runs with 16 ml each were necessary to process the entire sample. After sample application, the column was developed with a linear/isocratic gradient of 0–0.2 M-NaCl in equilibration buffer. As shown in Fig. 2, the trypsin-inhibitory activity is resolved into two main peaks. The activity eluted in fractions 25–40 (0.06–0.09 M-NaCl), inhibitory towards elastase, contains α_1 -PI, and the activity in fractions 47–59 (0.1–0.12 M-NaCl), which is inactive towards elastase, corresponds to RPI I. The antichymotryptic-activity profile, not included in the Figure, closely followed that of the antitryptic activity.

Step 3b: anion-exchange chromatography on Mono Q (RPI II). RPI II, eluted in peak 2 during Affi-gel Blue chromatography (Fig. 1) was further purified by chromatography on Mono Q. The pooled Affi-gel Blue fractions, concentrated to 15 ml, were directly applied to a Mono Q column, equilibrated with 20 mM-Bistris/HCl buffer, pH 6.0. A linear/isocratic salt gradient of 0–0.2 M-NaCl in the buffer was then initiated. As depicted in Fig. 3, the trypsin-inhibitory activity is eluted as a single peak at 0.09–0.12 M-NaCl.

The fractions from steps 3a and 3b, containing the three inhibitors, were pooled separately and concentrated, each to a volume of 2 ml, by ultrafiltration.

Step 4: recycling chromatography on Sephadex G-75 (superfine grade) (α_1 -PI, RPI I and RPI II respectively). Final purification of inhibitors was by gel-exclusion chromatography on Sephadex G-75 (superfine grade). In preliminary experiments it was found that after a single chromatographic run on Sephadex G-75 (or other gels with a similar fraction range) the inhibitor preparations were not homogeneous, as judged by analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This drawback could be overcome by applying the recycling technique (Porath & Ben-nich, 1962). The concentrated inhibitor samples (2 ml) from the previous step were applied to a column of Sephadex G-75, equilibrated with 25 mM-Bistrispropane/HCl buffer, pH 6.7, containing 50 mM-NaCl. After four cycles, the inhibitors were eluted as single peaks of activity (results not shown). The respective inhibitor fractions were pooled, concentrated to a small volume and stored at -20°C until further use.

Fig. 2. Separation of α_1 -PI from RPI I by ion-exchange chromatography on Mono Q

The concentrated dialysed sample from DEAE-Sephacel (step 2) was loaded into the Pharmacia FPLC-system superloop, and a 16ml portion was applied to the Mono Q column (0.5cm $\times$ 10cm) equilibrated with 20mM-Bistris/HCl buffer, pH6.0. Starting at fraction 17, the column was eluted with a linear/isocratic gradient of 0–0.2M-NaCl in the buffer. The flow rate was 1 ml/min, and 1 ml fractions were collected at 4°C ambient temperature. After completion of the first run and re-equilibration of the column with starting buffer, the second 16ml portion was chromatographed in an identical fashion. The Figure depicts one of two consecutive runs. -----, Concn. of NaCl; —, A_{280} ; ●, antitrypsin activity; ○, anti-elastase activity.

Fig. 3. Ion-exchange chromatography of RPI II on Mono Q

The concentrated peak 2 activity (15ml) from Affi-gel Blue chromatography was applied to a Mono Q column, equilibrated with 20mM-Bistris/HCl buffer, pH6.0. Starting at fraction 16, the column was developed with a linear/isocratic gradient of 0–0.2M-NaCl in the buffer. Other conditions were as given in the legend to Fig. 2.

The purification scheme for the inhibitors is summarized in Table 2. On the assumption that recoveries were similar for the three inhibitors, their contributions to the antitryptic activity of crude rat serum can be estimated at about 68, 14 and 18% for α_1 -PI, RPI I and RPI II respectively.

Homogeneity and M_r values of inhibitors

Preparations of the inhibitors were homogeneous as judged by analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 4). Analysis of the results on double-logarithmic plots (Lambin, 1978) and comparison with standard proteins of known size indicated M_r values of 55000, 66000 and 65000 for α_1 -PI, RPI I and RPI II respectively.

Comparison of the antiproteinase activities of the three inhibitors

The activities of the inhibitors with three serine proteinases were compared and the results are summarized in Fig. 5. Trypsin is inhibited by each inhibitor at a molar ratio of close to 1:1 (Fig. 5a). As regards the interaction of α_1 -PI with the enzyme, the equivalence point is determined by extrapolation of the linear part of the curve to 100% inhibition. Deviation of inhibition curves from linearity is frequently observed (Koj & Regoeczi, 1981; Takahara & Sinohara, 1982) and is due to reasons detailed by Bieth (1980). Fig. 5(b) shows that the activity of chymotrypsin is completely inhibited by nearly equimolar concentra-

Table 2. *Purification of α_1 -PI, RPI I and RPI II*

For full experimental details see the text. Yield and purification values given are from a representative experiment.

Step		Antitrypsin activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Serum (20 ml)		808.7	940	0.86	100	1
Affi-gel Blue	Peak 1 (α_1 -PI, RPI I)	445.0	148	3.0	56	3.5
	Peak 2 (RPI II)	78.3	18	4.35	9.7	5.1
DEAE-Sephacel	(α_1 -PI, RPI I)	221.7	39	5.7	27	6.6
Mono Q	(α_1 -PI)	143.5	11	13.0	17.7	15.2
	(RPI I)	44.8	4.1	10.9	5.5	12.7
	(RPI II)	41.3	4.3	9.6	5.1	11.2
Sephadex G-75	(α_1 -PI)	81.3	4.5	18.1	10	21
	(RPI I)	16.7	1.1	15.2	2.1	17.6
	(RPI II)	21.0	1.4	15.0	2.6	17.4

Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis of purified proteinase inhibitors*

Electrophoresis was on 10–18% linear-gradient 10–18%-acrylamide slab gels, and sample preparation was as described by Rutschmann *et al.* (1982). Electrophoresis lasted for 20 h at a constant 10 mA. Gels were stained with 0.2% Serva Blue R in methanol/acetic acid/water (9:2:9, by vol.) and de-stained. Std, small- M_r standard protein mixture with (top to bottom) phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 21000) and lysozyme (M_r 14300); 1, purified RPI I, 8 μ g; 2, purified α_1 -PI, 8 μ g; 3, purified RPI II, 8 μ g.

tions of α_1 -PI and RPI I (1.1 and 1.2 mol of inhibitor/mol of proteinase respectively). In contrast, no inhibition by RPI II was detected under these conditions. Elastase was inhibited by α_1 -PI at a molar ratio of 1:1.3, but the other two inhibitors were without effect on this enzymic activity (Fig. 5c). The absence of inhibitory activity towards the proteinases mentioned appears to be an inherent property of the inhibitors rather than being a reflection of incubation times too short for inactivation of enzymes. This is concluded from control experiments demonstrating that inactivation of enzymes was completed within 5 min of incubation with inhibitors.

Immunological analysis of the inhibitors

In an attempt to define the immunological relationship between the three rat proteinase inhibitors, their diffusion patterns against a multivalent antiserum to rat serum proteins were examined by double-diffusion analysis (Ouchterlony, 1964). The results (not shown) indicated that α_1 -PI is antigenically different from both RPI I and RPI II. The finding that precipitin lines of RPI I and RPI II were fused, which suggests a close immunological relationship, was unexpected. Because of the similar M_r values of RPI I and RPI II and because both inhibitors are active towards trypsin, we cannot exclude the possibility that RPI I is contaminated with RPI II. Following from this, the alternative and most likely interpretation of the immunological data would be that the commercial antiserum contained antibodies against RPI II but not RPI I and that the observed precipitin lines are both due to RPI II, which would of course give a line of identity. This question remains to be investigated in more detail.

Fig. 5. Inhibition of trypsin (a), chymotrypsin (b) and elastase (c) as a function of the concentration of α_1 -PI (●), RPI I (○) and RPI II (Δ)

Trypsin (0.63 nmol of active enzyme), chymotrypsin (0.3 nmol of active enzyme) and elastase (0.3 nmol of active enzyme) were separately incubated with increasing amounts of the purified inhibitors for 15 min at 25°C, before addition of the appropriate substrate and determination of residual proteolytic activity.

Discussion

Our study demonstrates the presence of three functionally distinct trypsin-inhibitory activities in rat serum. Two of these inhibitors, α_1 -PI and RPI I, have properties suggesting them to be identical with the R_1 and R_2 components previously isolated from rat serum (Rosenberg *et al.*, 1976). First, α_1 -PI and the R_1 component are active against the three serine proteinases trypsin, chymotrypsin and elastase, whereas RPI I and the R_2 component are inhibitory towards trypsin and chymotrypsin but not elastase. Secondly, α_1 -PI and RPI I have estimated M_r values of 55000 and 66000 respectively, which are very similar to the values reported for the R_1 and R_2 components. Thirdly, α_1 -PI and RPI I, analogously to the situation observed for the R_1 and R_2 components, are antigenically distinct. The other trypsin inhibitor, designated RPI II, has an M_r value of 65000, as estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel filtration. This excludes the possibility that RPI II is homologous to α_1 -macroglobulin (Ganrot, 1973) or to α_1 -inhibitor III (Esnard & Gauthier, 1980) whose M_r values are greater than 200000. Further, antithrombin III was removed during the first purification step of RPI II. These data suggest that RPI II is different from any rat serum proteinase inhibitor so far described in the literature. On the other hand, RPI II seems to be homologous to contrapsin, a trypsin inhibitor that has been purified from mouse plasma (Takahara & Sinohara, 1982). This is concluded from the observations that (i) mouse contrapsin, like RPI II, is inhibitory towards tryp-

sin but does not affect the activity of chymotrypsin or elastase, (ii) in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis mouse contrapsin migrates as a molecule with an M_r of 64000, a value almost identical with that found for RPI II (65000), and (iii) neither inhibitor immunologically cross-reacts with α_1 -PI. A number of mammalian sera, such as those of man (Yoshida & Mega, 1979), rabbit (Koj *et al.*, 1978), dog (Abrams *et al.*, 1978), horse (Pellegrini & von Fellenberg, 1980), mouse (Takahara & Sinohara, 1982) and rat (Rosenberg *et al.*, 1976), have been reported to contain two or more forms of α_1 -PI, which can be separated by polyacrylamide-gel electrophoresis. Mouse and rat, however are unique among them, since their two isoforms do not cross-react immunologically, whereas those from the other species are immunologically identical.

Our data demonstrate that α_1 -PI is distinct from RPI I and RPI II by several criteria. In contrast, the relationship between RPI I and RPI II is less clear. The possibility that the inhibitory properties of RPI I are mimicked by the trypsin inhibitor RPI II on to which antichymotrypsin activity has become adsorbed during the fractionation of rat serum is very unlikely, since α_1 -antichymotrypsin was removed during Affi-gel Blue chromatography. Furthermore, RPI I has been shown to be identical with the previously reported R_2 component (Rosenberg *et al.*, 1976). The latter inhibitor was isolated by a procedure different from ours, and a specific antibody was raised against the protein. Therefore the more likely interpretation of our data would be that RPI I and RPI II, though

being distinct inhibitor species, have very similar physicochemical properties, which render a complete separation difficult. This would also explain why other investigators have failed so far to identify RPI II in the rat.

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