PURIFICATION AND SOME CHARACTERISTICS OF TWO HUMAN SERUM PROTEINS INHIBITING PAPAIN AND OTHER THIOL PROTEINASES

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1. Introduction

Human serum contains 7 well characterized proteinase inhibitors with more or less broad specificity to serine proteinases [1,2]. In addition, two inhibitors not inhibiting serine proteinases have been described: β_1-collagenase inhibitor [3] and α_2-thiol proteinase inhibitor [4]. The partially purified α_2-thiol proteinase inhibitor has been shown not to be identical with the known inhibitors of serine proteinases. Its properties are also quite different from those of the human epidermal thiol proteinase inhibitor, purified in our laboratory [5,6].

Here I describe the purification of two thiol proteinase inhibitors with α_1- and α_2-mobilities from human serum. The purified inhibitors resemble each other in their immunological properties and inhibiting spectra, but have different charges and molecular sizes. The inhibitor with α_2-mobility seems to be identical with the thiol proteinase inhibitor in [4]. A preliminary note of this work has been published [7].

2. Materials and methods

Normal human serum was prepared from the blood of healthy donors. The serum samples of at least 8 donors were pooled. Papain (for biochemical purposes) was obtained from E. Merck., ficin (crude), bromelain (grade II), bovine trypsin (type III) and chymotrypsin (type II) and porcine elastase (type I) were obtained from Sigma Chemical Co. Cathepsin B was prepared from human liver by the method in [8] and the epidermal thiol proteinase inhibitor and its antiserum as in [9]. Ampholine® carrier ampholytes were obtained from LKB. Rabbit antisera against human albumin, α_1-antitrypsin, α_1-antichymotrypsin, ceruloplasmin and Gc-globulins were obtained from Dako, rabbit antisera against inter-α-trypsin inhibitor, α_2-macroglobulin, antithrombin III, C_1-inactivator, α_1-acid glycoprotein, α_2-HS-glycoprotein, haptoglobin, β_1-C/β_1-A-globulins, α_2-PA glycoprotein and α_1-lipoprotein, and horse antiserum to whole human serum proteins were from Behringwerke. Papain–Sepharose 4B was prepared as in [9].

The inhibition of papain, ficin, bromelain, and cathepsin B was assayed using benzoylarginine-2-napthylamide, and inhibition of trypsin, chymotrypsin, and elastase using azocasein as a substrate [5]. The inhibition was expressed as the amount of an enzyme (mg) inhibited by 1 mg inhibitor (U/mg), or by 1 ml inhibitor solution (U/ml).

The protein concentrations were measured by the method in [10]. Protein solutions were concentrated by ultrafiltration on a Diaflo® UM-10 membrane (Amicon).

Rabbits were immunized 4 times intradermally with the purified inhibitor preparation (0.05 mg/ml) by the procedure in [7]. Immunodiffusion, immunoelectrophoresis, analytical electrofocusing (Ampholine pH 3.5–10) and preparative electrofocusing in sucrose gradient (Ampholine pH 4–6) were performed as in [5].

2.1. *Purification procedure*

All procedures were done in a cold room (+4°C).

1. *Acid treatment.* The serum was adjusted to pH 5.0 with 2 M HCl, the precipitated proteins were removed by centrifugation, and the supernatant was neutralized. The procedure removed

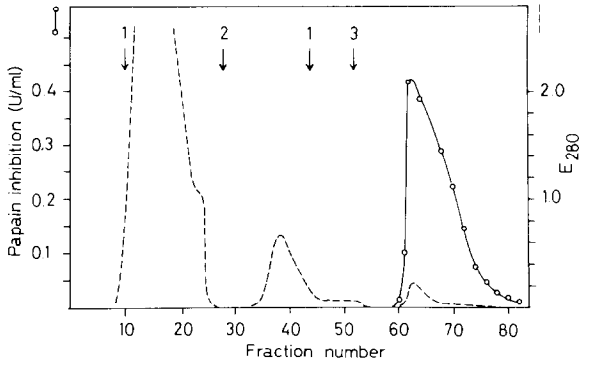

Fig.1. Papain–Sepharose affinity chromatography. At the points indicated by arrows, PBS (1), 4 M KSCN (2) and 20 mM Na_3PO_4, 0.1 M NaCl (3) were applied. Flowrate was 60 ml/h, and fraction vol. 5.5 ml. Fractions no. 62–73 were pooled.

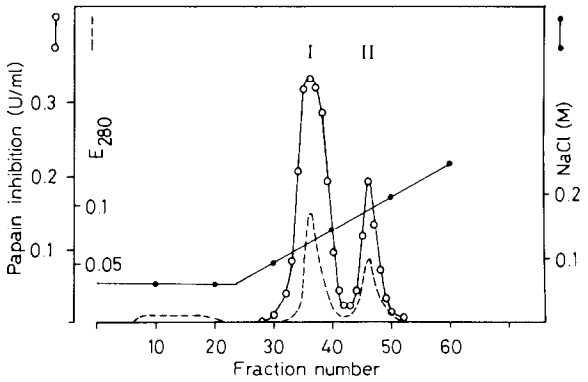

Fig.2. DEAE–Sephacel chromatography. The flowrate was 30 ml/h and the fraction vol. 5.5 ml. Inhibitor I (fractions no. 35–39) and inhibitor II (fractions no. 45–47) were pooled separately.

some material that was harmful in the following step.

2. *Papain–Sepharose chromatography (fig.1).* A 2.6 × 11 cm column of papain–Sepharose 4B was equilibrated with 10 mM phosphate buffer (pH 7.2) containing 0.145 M NaCl (phosphate-buffered saline, PBS). Acid-treated serum (60 ml) was applied to the column, unadsorbed proteins were washed out with PBS, unspecifically adsorbed proteins with 4 M KSCN dissolved in PBS, KSCN was washed out with PBS and the inhibitors were eluted with 20 mM Na_3PO_4, 0.1 M NaCl (pH 12.1).

2.3. *DEAE–Sephacel chromatography (fig.2)*

The pooled inhibitors from the preceding step were dialyzed against 20 mM Tris–HCl buffer, containing 50 mM NaCl, and applied to a 1.6 × 20 cm column of DEAE–Sephacel® (Pharmacia Fine Chemicals), equilibrated with the same buffer. The inhibitors were eluted with a linear gradient of NaCl (50–500 mM, total vol. 500 ml). Two inhibitor peaks, preparations I and II were fractionated and pooled separately.

2.4. *Sephadex G-150 chromatography*

The inhibitor preparations I and II were further fractionated in a 2.6 × 90 cm column of Sephadex G-150 (Pharmacia Fine Chemicals), equilibrated with 20 mM Tris–HCl buffer (pH 7.2) containing 50 mM NaCl.

3. Results

The purification procedure is summarized in table 1. Inhibitor I was 268-fold and inhibitor II 175-fold purified and the total inhibitor yield 12%, as compared to the activity of the acid-treated serum.

The molecular weight of inhibitor I was ~ 90 000 and that of inhibitor II ~ 175 000, as estimated by gel chromatography on Sephadex G-150. In preparative isoelectric focusing inhibitor I was focused at pH 4.6–4.7. During the focusing the inhibitor lost ~ 80% of its activity. The inhibitor II was focused at the steep pH front at the acidic end of the gradient (at pH 3.2–3.5). The heterogenity of inhibitor I was studied by analytical isoelectric focusing. Two distinct anodal bands were seen after staining of the gels with Coomassie brilliant blue or with the peri-

Table 1
Summary of the purification procedure

Step		Volume ml	Protein mg	Activity U/mg	Activity U
Acid-treated serum		60	4320	0.014	60.5
Papain–Sepharose		82	7.79	3.05	23.8
DEAE–Sephacel	I	33	2.94	3.82	11.2
	II	17	1.06	2.50	2.7
Sephadex G-150	I	37	1.48	3.75	5.6
	II	27	0.59	2.45	1.5

odic acid-Schiff procedure, suggesting that inhibitor I is a glycoprotein.

Inhibitors I and II had the same specificity against proteinases (fig.3): they strongly inhibited papain and ficin, cathepsin B and bromelain less intensively, and did not inhibit trypsin, chymotrypsin and elastase at all. The results suggest that the inhibitors are group-specific to thiol proteinases.

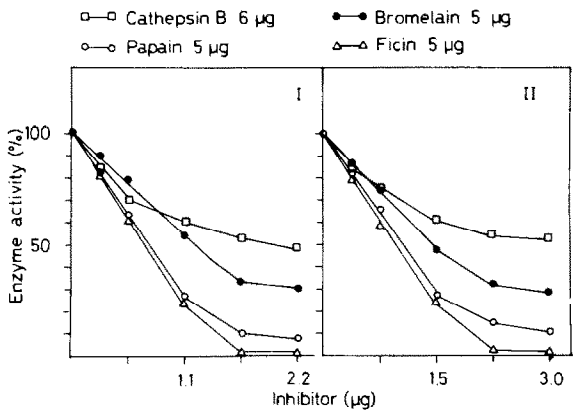

Fig.3. Inhibition of several thiol proteinases with the purified inhibitors I and II. Trypsin, chymotrypsin and elastase were not inhibited (not shown in the figure).

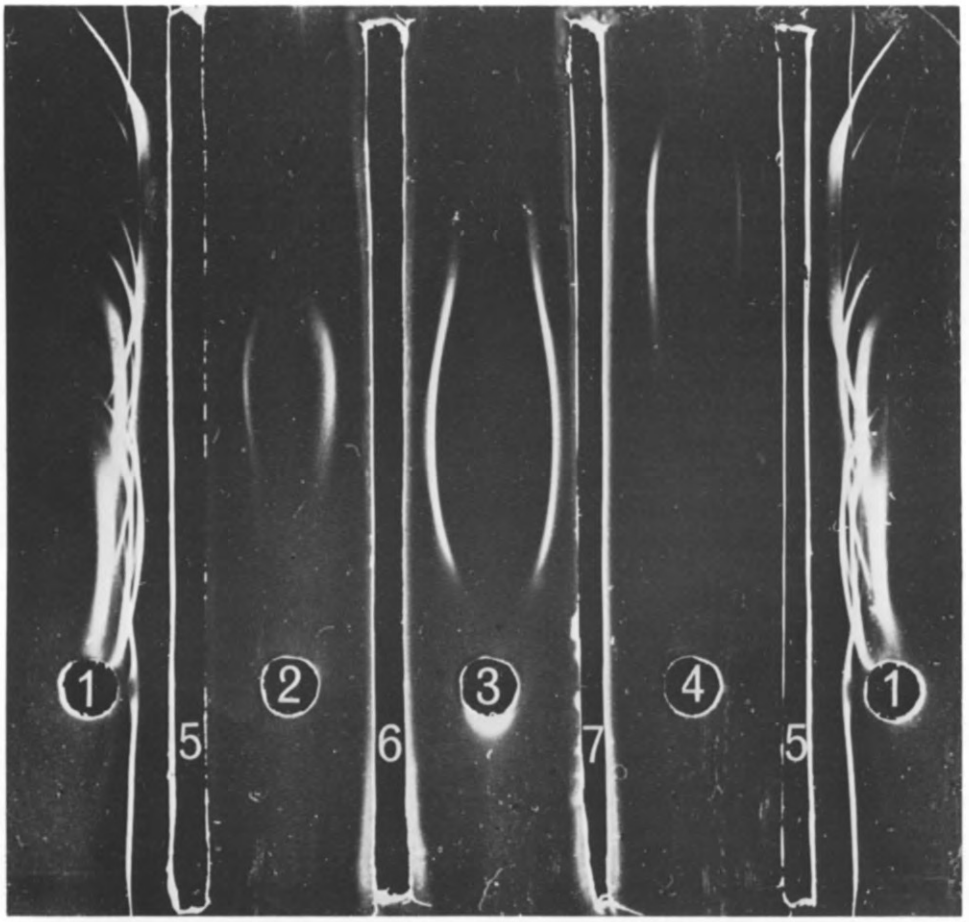

Fig.4. Immunoelectrophoresis of the purified inhibitors. (1) 1.25 μl of human serum; (2) 0.9 μg inhibitor I; (3) 10 μl human serum; (4) 0.9 μg inhibitor II; (5) 100 μl horse antiserum against human serum proteins; (6) 100 μl rabbit antiserum against inhibitor I; (7) 100 μl rabbit antiserum against inhibitor II.

Inhibitors I and II were immunologically closely related. In immunodiffusion, the antiserum raised against either of the inhibitors precipitated both inhibitor I and inhibitor II. One precipitation arch was also formed from human serum, and the precipitate arches from serum, inhibitor I and inhibitor II showed 'reaction of identity'.

In fig.4 results of an immunoelectrophoresis are presented. The precipitate arch of inhibitor II is located in the α_1- and that of inhibitor I in α_2-region. The corresponding arches were also formed from human serum, but no other serum proteins were precipitated.

Identification of the purified inhibitors with the known proteinase inhibitors and proteins of human serum was attempted by double radial immunodiffusion against those 15 specific antisera given in section 2. No one of those antisera precipitated any material in inhibitor preparations I and II. In addition, the purified inhibitors did not crossreact with the antiserum to the epidermal thiol proteinase inhibitor, and vice versa, the antisera against the serum inhibitors did not precipitate the epidermal inhibitor.

4. Discussion

The simple purification procedure described above yielded two proteins having similar immunological properties and inhibiting capacities against thiol proteinases. However, they have marked differences in their molecular sizes and charges: inhibitor II is about twice as large as inhibitor I and has a more negative charge and higher electrophoretic mobility than inhibitor I. An explanation to these differences may be that inhibitor II is a dimer of inhibitor I, or inhibitor I is a cleavage product of inhibitor II. Inhibitor I seems to be the major form of thiol proteinase inhibitor in fresh human serum (cf. fig.2,4) and is probably identical with the α_2-thiol proteinase inhibitor in [4].

The poor inhibition of cathepsin B by the serum thiol proteinase inhibitors makes it difficult to schedule their physiological function. Probably the enzyme affected by them is not cathepsin B, but may be one of the other known thiol proteinases of human tissues, e.g., cathepsin C [11], H [12,13], L [12,14] or collagenolytic cathepsin [15].

These data suggest that the human serum thiol proteinase inhibitors are not identical with other proteinase inhibitors of serum or with the epidermal thiol proteinase inhibitor. The availability of specific antiserum makes it possible to quantitate the total amount of the inhibitors by simple radial immunodiffusion, or both of them separately by quantitative crossed immunoelectrophoresis.

Recently, a thiol proteinase inhibitor has been purified from human serum [16]. The α_1- and α_2-proteins were not separated and the inhibitor is called 'α-thiol proteinase inhibitor'. Our results are in good agreement.

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